Université de Sherbrooke

Role of NLRX1 in glutamate uptake and release by astrocytes

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Mémoire présenté à la Faculté de médecine et des sciences de la santé en vue de l'obtention du grade de maitre ès sciences (M. Sc.) en immunologie

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This thesis is dedicated to

My beloved husband and my sweet son who were always by my side giving me power, support, and love to overcome all the challenges though out my way to achieve this work,

My dearest, Mother and sisters who were always a source of love, motivation and confidence

RÉSUMÉ

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Le glutamate est le principal neurotransmetteur excitateur du système nerveux central (SNC). Lorsque le glutamate est libéré dans la fente synaptique par les neurones présynaptiques, seulement 20% du glutamate libéré est absorbé par les neurones postsynaptiques. Le glutamate restant dans l'espace extracellulaire doit être collecté puisqu'il peut être toxique pour les neurones. Une exposition excessive ou prolongée des neurones au glutamate induit une hyperstimulation des récepteurs du glutamate, conduisant éventuellement à la mort neuronale dans le cadre d'un processus appelé « excitotoxicité du glutamate ».

Les astrocytes sont les cellules qui absorbent la majorité du glutamate extracellulaire grâce à leur expression de transporteurs d'acides aminés excitateurs 1 et 2 (EAAT1 et EAAT2). Les astrocytes absorbent le glutamate contre le gradient de concentration, ce qui demande un taux élevé d'ATP intracellulaires. Un haut taux de stress oxydatifs inhibe l'absorption du glutamate.

Les astrocytes libèrent également des traces de glutamate, par exocytose médiée par le Ca^{2+}, dans l'espace extracellulaire, ce qui permet de synchroniser les neurones adjacents. Toute anomalie astrocytaires entraîne une absorption réduite ou une libération excessive de glutamate prédisposant à une excitotoxicité du glutamate. Ces anomalies sont présentes dans plusieurs troubles du SNC.

NLRX1 est un capteur immunitaire inné de la famille des récepteurs de type NOD. Il s'agit d'une molécule anti-inflammatoire qui inhibe les voies de signalisation telles que NF-κB et RIG-1-MAVS. NLRX1 est aussi considéré un facteur de survie puisqu'il inhibe la mort neuronale. Contrairement aux autres NLRs, NLRX1 est localisé dans les mitochondries. NLRX1 augmente la fission mitochondriale et la production d'ATP et il inhibe le stress oxydatif.

Par conséquent, nous avons émis l'hypothèse qu'en renforçant les fonctions mitochondriales dans les astrocytes, NLRX1 améliore l'absorption du glutamate et inhibe la libération de glutamate par les astrocytes. Pour répondre à notre hypothèse, nous avons étudié le rôle de NLRX1 dans la capture et la libération du glutamate par des cultures d'astrocytes primaires de souris WT et *Nlrx1*$^{-/-}$ et les mécanismes par lesquels NLRX1 atténue ses effets. Nos résultats ont révélé que NLRX1 potentialise la capture de glutamate astrocytaire en augmentant la production d'ATP mitochondriale et en prévenant le stress oxydatif, qui à leur tour maintiennent les fonctions saines de EAAT1 et EAAT2 dans les astrocytes. NLRX1 supprime également la libération de glutamate astrocytaire en réprimant la libération de Ca^{2+} du réticulum endoplasmique (ER), ce qui supprime l'exocytose du glutamate médiée par le Ca^{2+}. Ensemble, nos données suggèrent que NLRX1 est un régulateur potentiel de l'homéostasie du glutamate dans le SNC.

Mots-clés: NLRX1, absorption de glutamate, libération de glutamate, astrocytes, excitotoxicité.

SUMMARY

Role of NLRX1 in glutamate uptake and release by astrocytes

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Thesis presented to Faculty of Medicine and Health Sciences for the obtention of a Master's degree (M.Sc.) in Immunology, Faculty of medicine and health sciences, Université de Sherbrooke, Sherbrooke, Québec, Canada, J1H 5N4

Glutamate is the major excitatory neurotransmitter in the central nervous system (CNS). When glutamate is released from the presynaptic neurons to the synaptic cleft, only 20% of the released glutamate is taken up by the postsynaptic neurons to transmit the excitatory signals. The remaining glutamate (80% or more) must be collected from the extracellular space as it is potentially toxic to neurons. Excessive or prolonged exposure of neurons to glutamate induces hyperstimulation of the glutamate receptors in neurons that eventually leads to neuronal death in a process known as "glutamate excitotoxicity".

The process of glutamate uptake from the extracellular space is achieved mainly by astrocytes, which are the most numerous cell type in the CNS. Astrocytes express excitatory amino acid transporters 1 and 2 (EAAT1 and EAAT2) which are responsible for glutamate uptake. To uptake glutamate against its concentration gradient, EAAT1 and EAAT2 consume a high level of intracellular ATP. By contrast, oxidative stress inhibits their function of glutamate uptake.

In addition to glutamate uptake, recent studies demonstrate that astrocytes release traces of glutamate to the extracellular space, which helps synchronize and reinforce the firing of the adjacent neurons. Astrocytes release glutamate mainly by Ca^{2+}-mediated exocytosis. Any defect in astrocytic functions that results in reduced glutamate uptake and/or excess glutamate release predisposes to glutamate excitotoxicity, as occurs in many CNS disorders.

NLRX1 is an innate immune sensor from the non-inflammasome NOD-like receptors' family. It is known as an anti-inflammatory molecule that inhibits NF-κB and RIG-1-MAVS signaling pathways. NLRX1 also acts as a survival factor that inhibits neuronal death. Unlike other NLRs, NLRX1 is localized to the mitochondria and was shown to enhance mitochondrial fission and ATP production while it inhibits oxidative stress.

Therefore, we hypothesized that by enhancing mitochondrial functions in astrocytes, NLRX1 enhances glutamate uptake and inhibits glutamate release by astrocytes. To address our hypothesis, we investigated the role of NLRX1 in glutamate uptake and release by primary astrocyte cultures from WT and *NLRX1*$^{-/-}$ mice and the mechanisms by which NLRX1 mediates its effects. Our results revealed that NLRX1 potentiates astrocytic glutamate uptake by enhancing mitochondrial ATP production and preventing oxidative stress, which in turn maintain healthy functions of EAAT1 and EAAT2 in astrocytes. NLRX1 also suppresses astrocytic glutamate release by repressing Ca^{2+} release from the endoplasmic reticulum (ER), which in turn suppresses Ca^{2+}-mediated glutamate exocytosis. Taken together, our data suggest that NLRX1 is a potential regulator of glutamate homeostasis in the CNS.

Keywords: NLRX1, glutamate uptake, glutamate release, astrocytes, excitotoxicity.

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LIST OF ABBREVIATIONS

$[Ca^{2+}]_i$	Intracellular Ca^{2+}
µM	Micromolar
2-APB	2-Aminoethyl diphenylborinate
AA	Arachidonic acid
AAT	Aspartate aminotransferase
ACR	Acute cellular rejection
AD	Alzheimer's disease
AD domain	Acidic transactivation domain
ALS	Amyotrophic lateral sclerosis
AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-propionate
APCs	Antigen-presenting cells
ATN	Acute tubular necrosis
ATP	Adenosine triphosphate
BBB	Blood-brain-barrier
BCAT	Branched-chain aminotransferase
BDNF	Brain-derived neurotrophic factor
Best-1	Bestrophin-1
bFGF	Basic fibroblast growth factor
BIR	Baculovirus inhibitor of apoptosis protein repeat
CAPS	Cryopyrin-Associated Periodic Syndromes
CARD	Caspase recruitment domain
CIITA	Class II transactivator
CCI	Controlled cortical impact
CHB	Chronic hepatitis B
CLRs	C-type lectin receptors
CNS	Central nervous system
COPD	Chronic obstructive pulmonary disease
CsA	Cyclosporin A
DAMPs	Damage-associated molecular patterns
dbcAMP	Dibutyryl cyclic adenosine monophosphate

DCs	Dendritic cells
dFBS	Deactivated fetal bovine serum
DHK	Dihydrokainate
DHR	Dihydrorhodamine 123
DMSO	Dimethyl sulfoxide
dsRNA	Double-stranded RNA
EAAC-1	Excitatory amino acid carrier-1
EAATs	Excitatory amino acid transporters
EAE	Experimental autoimmune encephalomyelitis
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
FCCP	Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone
GABA	ɣ-aminobutyric acid
GDH	Glutamate dehydrogenase
GDNF	Glial-derived neurotrophic factor
GDNF	Glial cell line-derived neurotrophic factor
GLAST	Glutamate-aspartate transporter
GLT-1	Glutamate transporter-1
GPCRs	G protein-coupled receptors
GRP75	The chaperone glucose-regulated protein 75
H_2O_2	Hydrogen peroxide
HBSS	Hank's Balanced Salt Solution
HIV	Human immunodeficiency virus
HSP	Heat shock proteins
IBD	Inflammatory bowel disease
IFN	Interferon
IGF-1	Insulin-like growth factor-1
iGluRs	Ionotropic glutamate receptors
IP3	Inositol-1,4,5-trisphosphate
IRFs	Interferon regulatory factors
JNK pathway	JUN amino-terminal kinases-dependent pathway
KA	Kainic acid

LGP2	Laboratory of genetics and physiology 2
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
MAM	Mitochondria-associated membrane
MAVS	Mitochondrial antiviral signaling
MDA5	Melanoma differentiation associated gene 5
MDD	Major depressive disorder
MFN2	Mitofusin 2
mGluRs	Metabotropic glutamate receptors
mM	Millimolar
MS	Multiple sclerosis
mtDNA	Mitochondrial DNA
NBD	Nucleotide binding domain
NCM	Neuron-conditioned medium
NF-κB	Nuclear factor-κB
NH_3/NH_4^+	Ammonia/Ammonium
NLRs	NOD-like receptors
NLRX1	NOD-like receptor X1
Nlrx1$^{-/-}$	*Nlrx1* knockout
NLRX1-KD	NLRX1-knock down
NLRX1-KI	NLRX1-knock in
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NOS	Nitric oxide synthetase
O_2^-	Superoxide
OH.	Hydroxyl radicals
$OONO^-$	Peroxynitrite
$P2X_7$	P2X Purinoceptor 7
PACAP	Pituitary adenylate cyclase-activating polypeptide
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PD	Parkinson's disease

PGE	Prostaglandin E
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PRRs	Pattern recognition receptors
PYD	Pyrin domain
qPCR	Quantitative real-time PCR
RLRs	RIG-I-like receptors
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
Sc	Scrambled ShRNA
SEM	Standard error of the mean
SH group	Sulfhydryl group
SNAP25	Synaptosomal-associated protein 25
SNARE complex	Soluble N-ethylmaleimide-sensitive factor attachment protein receptors complex
ssRNA	Single-stranded RNA
TCA	Tricarboxylic acid
TG2	Transglutaminase type 2
THA	D,L-threo-hydroxyaspartate
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor-α
TUFM	Tu translation elongation factor
VAMP2	Vesicle-associated membrane protein 2
V-ATPase	Vacuolar (H+) ATPase
VGLUT1 and VGLUT2	Vesicular glutamate transporters 1 and 2
VRACs	Volume-regulated anion channels
WT	Wild-type

1. INTRODUCTION

1.1. The Central Nervous System and its cellular components

The central nervous system (CNS) consists of the brain and the spinal cord. It is the fascinating system in our body that controls all our voluntary and involuntary movements, sensations and higher cognitive functions such as the memory, emotions, thinking, learning…. etc. It also controls our internal body functions including, but not restricted to the heart rate, respiration, and digestion. In order to perform its highly integrated functions, the CNS contains two major types of cells: neurons and glial cells, which include astrocytes, oligodendrocytes, and microglia.

1.1.1. Neurons and neurotransmitters

Neurons are the main functional unit in the CNS. They are responsible for the transmission of signals from the body to the CNS and vice versa. The structure of neurons is divided into three main parts, which are: the cell body, the dendrites, and the axon **(Figure 1)**. The neuronal cell body is the central part of the neuron that contains the nucleus and all the organelles necessary for the proper neuronal function (Jacobson and Marcus 2008). Many projections arise from the cell body, that are called the dendrites. They are responsible for the transmission of signals from the presynaptic to the postsynaptic neurons at the regions of the synapses (Vanderah, Gould, and Preceded by (work): Nolte n.d.). The longest projection from the cell body is called the axon, that ends with the nerve terminal. The axon is covered by the myelin sheath, which is a lipid bilayer that helps to strengthen and speed up the signal transmission along the axon, through what is called the salutatory conduction (Jacobson and Marcus 2008). The fast propagating signals travel along the axons in the form of electrical impulses known as the "action potential", while the transmission from one neuron to the next occurs in a chemical form, through molecules known as "neurotransmitters" (Jacobson and Marcus 2008).

1.1.1.1. Functions of neurotransmitters

According to their functions, neurotransmitters could be excitatory, inhibitory or modulatory (Deutch 2013):

- Excitatory: These transmitters are likely to induce depolarization in the postsynaptic neuron and generate an action potential. Glutamate is the major excitatory neurotransmitter in the CNS. Epinephrine and norepinephrine are also common excitatory neurotransmitters.
- Inhibitory: Transmitters that are more likely to induce hyperpolarization in the postsynaptic neuron and inhibit the generation of the action potential. The most common inhibitory neurotransmitter in the brain is ɣ-aminobutyric acid (GABA), while glycine is the most common one in the spinal cord.
- Modulatory: These transmitters can modulate the action of other neurotransmitters. They diffuse to affect a larger number of neurons than the excitatory or the inhibitory neurotransmitters; however, they conduct a slower effect.

1.1.1.2. Types of neurotransmitters

According to the structure of neurotransmitters (Deutch 2013), they are classified into:

- Amino acids, such as:

  Glutamate: The predominant excitatory neurotransmitter in the CNS that plays a role in cognitive functions such as memory and learning.

  GABA: Inhibitory neurotransmitter that is involved in vision, motor control, and the regulation of anxiety.
- Acetylcholine: plays a role in both the central and the peripheral nervous systems. In the CNS, it is involved in cognitive functions such as memory and learning.
- Monoamines: examples are:

  Epinephrine and norepinephrine: play a role in the alertness in cases of dangers or stress.

  Serotonin: participates in the regulation of the mood, sleep, anxiety, sexuality, and appetite.

  Dopamine: plays a major role in the coordination of body movements.
- Peptides: include **oxytocin**, which plays a role in social recognition, bonding, and sexual reproduction, and **endorphins**, that inhibit pain signals and stimulate the feeling of euphoria.
- Purines: such as **adenosine** and **adenosine triphosphate (ATP)**.
- Gas transmitters: involving **nitric oxide** and **carbon monoxide**.

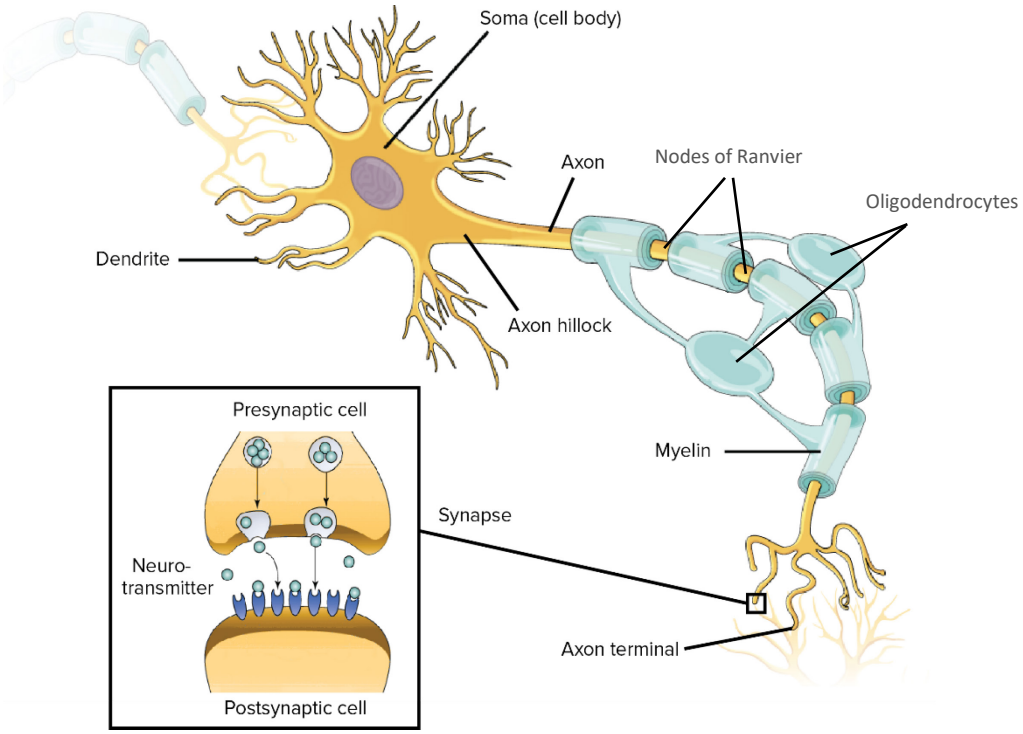

Figure 1: Structure of neurons and synapses. Representative scheme of a typical neuron containing the cell body (soma) with the dendrites and the axon covered by myelin and ends with the axon terminal. The detailed structure of the synapse is presented on the left side of the figure. It shows the presynaptic and the postsynaptic neurons, with space in between known as the synaptic cleft where the neurotransmitter is released. *(Untitled image licensed by Khan Academy under CC-BY-NC-SA and is available for free at www.khanacademy.org).*

1.1.2. Oligodendrocytes

They represent a type of glial cells in the CNS. The main function of oligodendrocytes is to form the myelin sheath around the axons (Jacobson and Marcus 2008). Myelin is a lipid layer that is formed by a sheet of the oligodendrocyte's plasma membrane, and a single oligodendrocyte can form myelin sheaths for several axons. Myelin sheath has a protective role for the axon against degeneration (Fitzner et al. 2006). It also wraps the axon in a non-continuous manner, leaving bare areas of the axon known as the "nodes of Ranvier". As the action potential is traveling along the axon, the electrical impulses jump across the nodes of Ranvier, which helps to accelerate and reinforce the signal transmission (Byrne and Roberts 2009). In addition to myelin formation, oligodendrocytes release several neurotrophic factors such as glial-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), and insulin-like growth factor-1 (IGF-1) (Bradl and Lassmann 2010).

1.1.3. Microglia

They are the professional innate immune cells in the CNS. Microglia represent the tissue-resident macrophages, and they express the specific macrophage markers such as CD11b, CD14, CSF1R, CD80/86, and MHC-II (Graeber and Streit 2010; Kettenmann et al. 2011; Saijo and Glass 2011). They represent 10 to 15% of the total glial cells in the CNS (Nayak, Roth, and McGavern 2014) and they are found in the brain, spinal cord, retina and optic nerve (Goldmann and Prinz 2013). Unlike other glial cells, they possess a characteristic morphology. In the resting state, they are highly ramified cells which allow them to scan the environment of the CNS for the presence of pathogens or damaged tissue, with the alternative extension and retraction of their ramifications. When microglia become activated, they retract their ramifications and acquire a rounded or ameboid shape (Nayak et al. 2014). Microglia play a role in neurogenesis, neuroprotection, and synapse formation, in addition to their innate immune functions (Shastri, Bonifati, and Kishore 2013). Like macrophages, microglia function as phagocytic cells and antigen-presenting cells on MHC-II (Nayak et al. 2014; Shastri et al. 2013). In case of CNS pathology, microglia are classically activated to the inflammatory M1 phenotype, which release various pro-inflammatory molecules such as IL-1β, TNF-α, IL-6, CCL5, ROS, NO, iNOS, that skew the adaptive immune response towards the inflammatory phenotypes. By contrast, if microglia are alternatively activated to M2 subtype, they release regulatory cytokines such as IL-10 and TGF-β that inhibit inflammation and promote tissue repair (Goldmann and Prinz 2013; Kettenmann et al. 2011).

1.1.4. Astrocytes

They are the most plentiful cell type in the CNS, as their number exceeds the number of neurons by more than 5-folds (Liu et al. 2018). Astrocytes are star-shaped cells that connect to each other by gap junctions. They also form extensive connections with the blood vessels and neurons; therefore, they coat the CNS in a continuous and non-overlapping mechanism (Sofroniew and Vinters 2010).

According to their morphology, astrocytes are classified into two main types, protoplasmic and fibrous astrocytes **(Figure 2)**. Protoplasmic astrocytes are found in the gray matter, and they were shown to envelop synapses. They exhibit a characteristic morphology with numerous short fine branches. Fibrous astrocytes are present in the white matter, and they are characterized by

their long fiber-like processes, by which they connect to the nodes of Ranvier (Sofroniew and Vinters 2010).

Astrocytes perform several vital functions in the CNS that maintain the homeostasis and ensure proper neuronal functions. During ontogeny, astrocytes play an essential role in the development of both white and gray matters. They form molecular boundaries and release several mediators such as "thrombospondin", that guide the formation and growth of axons and synapses (Christopherson et al. 2005; Powell and Geller 1999). Through their connections to the blood vessels, astrocytes control the amount of blood flow to neurons, by releasing molecular mediators such as prostaglandin E (PGE), nitric oxide (NO) and arachidonic acid (AA), that modulate the diameter of the blood vessel according to the neuronal activity (Gordon, Mulligan, and MacVicar 2007). Astrocytes also uptake glucose from the blood, convert it to lactate and provide it to neurons as a source of energy (Suh et al. 2007). Additionally, they store glycogen, and by glycogenolysis, they maintain the energy support to the neurons in case of hypoglycemia or increased neuronal activity (Pellerin et al. 2007). Moreover, Astrocytes' end-feet support the blood-brain-barrier (BBB) and maintain its integrity (Abbott, Rönnbäck, and Hansson 2006). The BBB is a diffusion barrier that protects the CNS from the blood-born infections and harmful substances. It is formed by the cerebral capillary endothelial cells, capillary basement membrane, perivascular pericytes, and astrocytes' end-feet (Ballabh, Braun, and Nedergaard 2004). Through astrocytes' ion channels (such as Ca^{2+} and K^{+}), aquaporin-4 water channel, and Na^{+}/H^{+} exchanger, astrocytes maintain the ionic and fluid balance and regulate the pH in the CNS, respectively (Obara, Szeliga, and Albrecht 2008; Simard and Nedergaard 2004). They also play a very important role in the homeostasis of neurotransmitters. Astrocytes uptake the excess synaptically-released neurotransmitters such as glutamate, GABA, and glycine from the extracellular space, metabolize them using specific enzymes expressed by astrocytes and release their metabolites back to the extracellular space, where they are taken up by neurons to resynthesize active neurotransmitters (Mahmoud et al. 2019; Sofroniew and Vinters 2010). In addition to the uptake of neurotransmitters, astrocytes release traces of transmitters, known as "Gliotransmitters" such as glutamate, GABA, D-serine and ATP to the adjacent neurons, which help to synchronize and strengthen their firing (Hamilton and Attwell 2010; Mahmoud et al. 2019). Moreover, astrocytes release many growth factors and neuroactive steroids such as estradiol and progesterone to maintain normal synaptic functions (Barres 2008; Garcia-Segura and Melcangi 2006). In CNS pathologies, astrocytes act as innate

immune cells that release several cytokines such as IL-6, IL-1ß, TNF-α, TGF-ß, IL-12, and IL-23 and chemokines such as CCL2, CCL5, CXCL10, CXCL12, CXCL8, and CXCR5 which reactivate and recruit peripheral and resident immune cells to the site of the lesions. Like microglia, activated astrocytes can present antigens on MCH-II, in addition to their limited phagocytic function (Farina, Aloisi, and Meinl 2007).

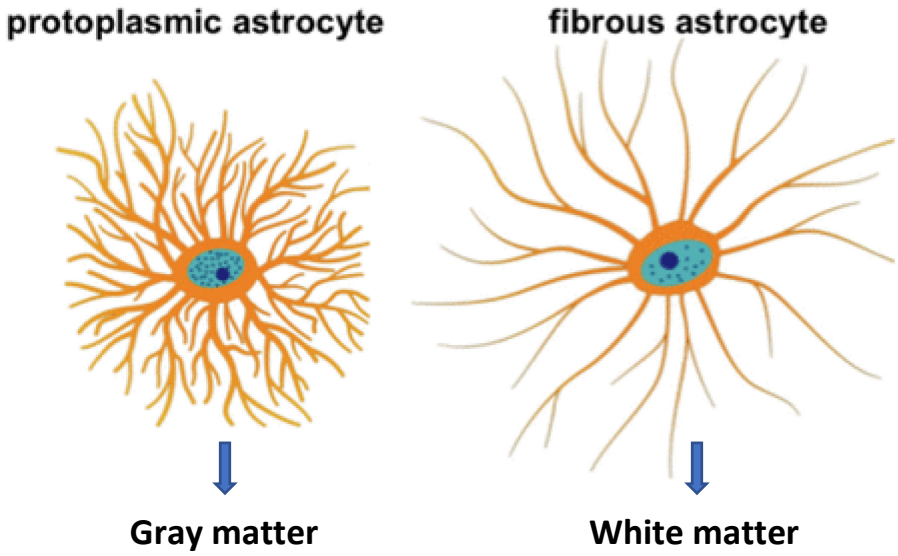

Figure 2: Morphological difference between protoplasmic and fibrous astrocytes. Scheme showing the morphological difference between protoplasmic astrocytes in the grey matter with their short fine branches (left side) and fibrous astrocytes in the white matter with their long fiber-like processes (right side). *Figure modified from (Ingo et al. 2018).*

1.2. Glutamate uptake by astrocytes in the CNS

This section is modified from my published review (Mahmoud et al. 2019).

Although glutamate is the predominant excitatory neurotransmitter in the CNS (Fonnum 1984), excess glutamate in the extracellular space is potentially toxic to neurons. Therefore, proper uptake of the excess synaptically-released glutamate from the extracellular space is indispensable and is performed by all cells in the CNS, including astrocytes, microglia, oligodendrocytes, and neurons (Mahmoud et al. 2019). Astrocytes are the principal cells responsible for glutamate uptake as they uptake about 90% of the released glutamate in the CNS (Eulenburg and Gomeza 2010; Lehre and Danbolt 1998).

1.2.1. Excitatory amino acid transporters (EAATs)

The process of glutamate uptake is mediated by Na^+-dependent glutamate uptake transporters, known as "excitatory amino acid transporters" (EAATs). Five isoforms of EAATs were identified so far, which are EAAT1 and EAAT2 in human and known in murine animals as glutamate-aspartate transporter (GLAST) and glutamate transporter-1 (GLT-1), respectively. Together EAAT1 and EAAT2 (GLAST and GLT-1) represent the majority of EAATs in the CNS, and they are expressed mainly by astrocytes. They uptake 80-90% of the total extracellular glutamate in the CNS (Anderson and Swanson 2000; Rose et al. 2018). Other isoforms include EAAT3, also known as excitatory amino acid carrier-1 (EAAC-1) and is expressed by neuronal cell bodies (Kanai and Hediger 1992), EAAT4 expressed by cerebellar Purkinje cells (Fairman et al. 1995), and EAAT5 expressed by retinal glial cells (Arriza et al. 1997).

1.2.2. Expression profile of EAAT1 and EAAT2

EAAT1 and EAAT2 share the same structure with 65% similarity in their amino acid components (Gegelashvili and Schousboe 1997). They also have the same affinity to glutamate, and both can reduce extracellular glutamate to the same level (Owe, Marcaggi, and Attwell 2006). However, they differ in their anatomical distribution in the CNS.

EAAT1 (GLAST) predominates in the CNS early after birth and is expressed by radial glial cells and immature astrocytes (Shibata et al. 1997). In adult CNS, EAAT1 (GLAST) predominates in the cerebellum, Müller glia in the retina, and cochlear glial cells in the inner ear, with limited expression in the forebrain. In adulthood, EAAT1 is expressed primarily by astrocytes, with some limited expression by microglia and oligodendrocytes (Schmitt et al. 1997).

On the other hand, EAAT2 (GLT-1) represents the major EAAT in the adult CNS. It acquires its maximum expression within 3-5 weeks after birth. Unlike EAAT1, EAAT2 predominates in the forebrain and represents the main EAAT expressed by mature astrocytes in the CNS (Furuta, Rothstein, and Martin 1997; Ullensvang et al. 1997).

In astrocytes, EAAT1 and EAAT2 are distributed in the form of clusters on the astrocytic processes in contact with active glutamatergic neurons (Zhou and Sutherland 2004). Moreover, the expression of both EAATs in astrocytes positively correlates with increased neuronal activity and its associated increased glutamate release (Poitry-Yamate, Vutskits, and Rauen 2002).

1.2.3. Evidence that EAAT2 and EAAT1 play the major role in glutamate uptake in the CNS

Many experimental studies revealed the importance of EAAT-2 and EAAT-1 in the clearance of glutamate and prevention of its toxic effect on neurons. A study on astrocyte-deficient neuronal cultures reported a 100-fold increase in neuronal death mostly by glutamate toxicity (Rosenberg and Aizenman 1989). Other studies on preparations from the hippocampus and cerebellum showed that glutamate release from glutamatergic neurons induced glutamate transporter-associated currents in astrocytes, while no similar currents were detected in the excitatory neurons. These currents were abolished upon using D,L-threo-hydroxyaspartate (THA, a nonselective EAAT inhibitor) or dihydrokainate (DHK, a GLT-1 selective inhibitor) and in GLT-1 knockout mice (Clark and Barbour 1997; Kojima et al. 1999).

In vivo studies on GLAST and GLT-1 double knockout mice revealed the development of neurodegeneration and progressive paralysis in these mice (Rothstein et al. 1996). Regarding the preferential importance of EAAT2 (GLT-1) in glutamate uptake, GLT-1 deficient mice, as well as mice lacking GLT-1 exclusively in astrocytes in a conditional knockout mouse model showed higher susceptibility to lethal seizures and loss of neurons (Petr et al. 2015; Tanaka et al. 1997), while GLAST-mutant mice were presented with cerebellar injury that resulted in defective motor coordination (Watase et al. 1998). By contrast, EAAT3 and EAAT4 knockout mice showed only minor neurological deficits (Huang and Bergles 2004; Peghini, Janzen, and Stoffel 1997), while no EAAT5 knockout animal model has yet been reported.

1.2.4. Mechanism of glutamate uptake by EAATs

When glutamate is released from the excitatory pre-synaptic neurons, a small percentage of this glutamate is taken up by the post-synaptic neurons (20% in the retina and cerebellar cortex, and a less percentage in the hippocampus), which is enough to perform the function of excitatory transmission. The majority of the released glutamate (80% or more) is taken up by EAAT1 and EAAT2 in astrocytes **(Figure 3A)** (Anderson and Swanson 2000; Mahmoud et al. 2019).

Normally, glutamate concentration in the extracellular space is in the millimolar range (mM) while its concentration intracellularly is in micromolar quantities (μM). To achieve glutamate uptake against its concentration gradient, astrocytes consume high levels of intracellular adenosine triphosphate (ATP) generated by activation of the Na^+/K^+ ATPase in astrocytes (Pellerin and

Magistretti 1997); therefore, glutamate uptake is considered one of the highest energy-consuming processes in the CNS (Anderson and Swanson 2000).

Several previous studies described the stoichiometry driving glutamate uptake by astrocytes to be achieved by the inward transport of 3 Na^+ and 1 H^+ ions with each glutamate anion taken up, in exchange with the outward transport of 2 K^+ ions, with their concentration gradients (Levy, Warr, and Attwell 1998). This process is associated with what is called "uptake current", which is a form of membrane depolarization in astrocytes caused by the inward transport of 2 additional positive ions (Bowman and Kimelberg 1984).

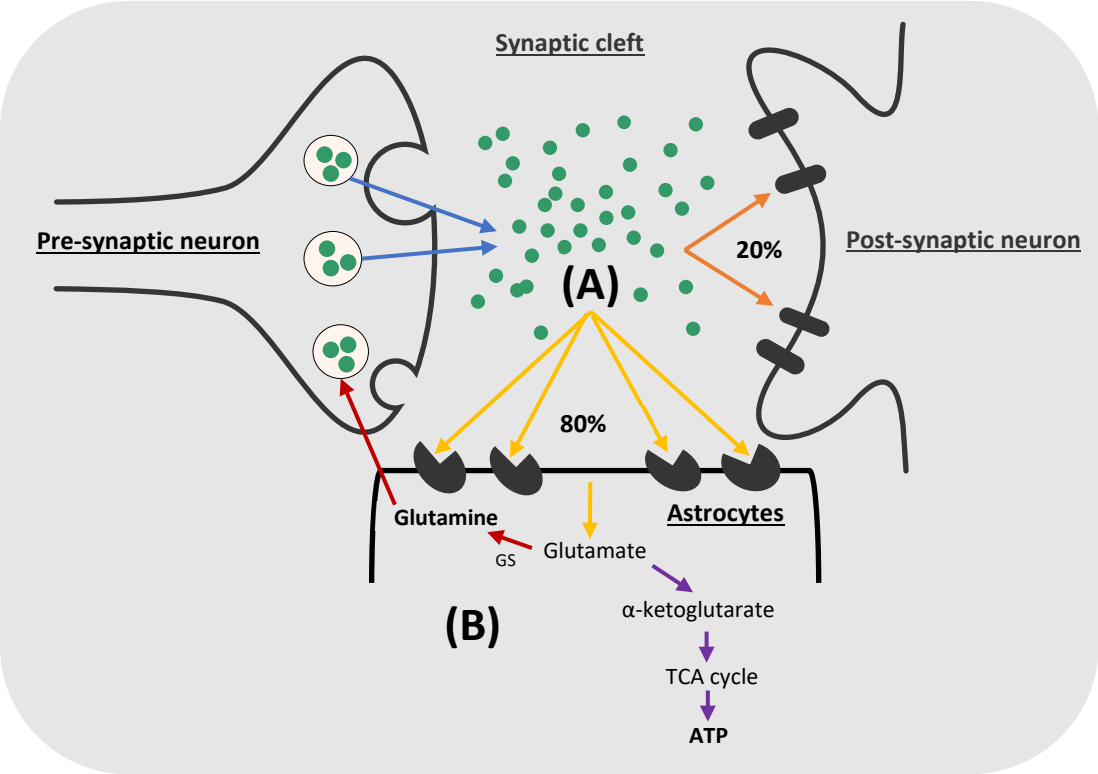

Figure 3: Glutamate uptake and metabolism by astrocytes. (A) Glutamate uptake by astrocytes: only 20% of the released glutamate at the synapses is transferred to the post-synaptic neurons while the remaining extracellular glutamate (80%) is taken up by EAAT2 and EAAT1 in astrocytes. **(B)** Glutamate metabolism in astrocytes: glutamate can be metabolized by glutamine synthetase enzyme to glutamine which is released back to neurons or could be metabolized to α-ketoglutarate to be used in ATP synthesis (Mahmoud et al. 2019).

1.2.5. Metabolism of glutamate in astrocytes

Glutamate metabolism in astrocytes follows one of two main pathways as shown in **(Figure 3B)** (Mahmoud et al. 2019). Via glutamine synthetase enzyme, which is preferentially expressed

in astrocytes, glutamate is converted to glutamine (Waniewski and Martin 1986), which is then released from astrocytes to the extracellular space by the Na^+-dependent, electroneutral transporter SN1. Neurons then uptake glutamine from the extracellular space by their system A transporters and use it to resynthesize active neurotransmitters such as glutamate and GABA (Bröer et al. 2002; Bröer and Brookes 2001). Interestingly, activation of glutamine synthetase enzyme also has a detoxifying effect in the CNS, as it breaks down the blood-derived ammonia and brain ammonium (NH_3/NH_4^+), keeping their concentrations less than 0.1 mM which protects against ammonium neurotoxicity (Marcaggi and Coles 2001).

On the other side, glutamate can undergo oxidative degradation to produce α-ketoglutarate, which enters the tricarboxylic acid (TCA) cycle and represents a precursor for ATP production. Oxidative metabolism of glutamate is achieved either by deamination, via the mitochondrial enzyme, glutamate dehydrogenase (GDH), or by transamination using aspartate aminotransferase (AAT), alanine aminotransferase, and branched chain aminotransferase (BCAT) enzymes (McKenna et al. 2016). Curiously, the oxidative metabolism of glutamate results in the production of an excessive amount of energy that compensates for the ATP originally consumed in glutamate uptake (McKenna 2013).

Depending on the concentration of glutamate in the extracellular space, glutamate metabolism is directed to one of these two pathways. If glutamate concentration is less than 0.2 mM, glutamate metabolism is directed towards glutamine synthesis to provide sufficient glutamate for neurons, while if it is more than 0.2 mM, glutamate is oxidatively degraded to maintain enough amount of ATP for glutamate uptake (McKenna et al. 1996).

1.2.6. EAAT1 and EAAT2 regulation of expression

Many factors regulate the expression of EAAT-1 and EAAT-2 under physiological and pathological conditions, at the level of the gene transcription and translation, post-translation, as well as the transporter activity. Here, we summarize the important factors at different levels.

1.2.6.1. Transcriptional and translational modifications

Glutamate concentration in the extracellular environment is one of the essential factors that influence the expression of glutamate transporters in astrocytes, as increased glutamate concentration was shown to upregulate GLAST protein expression and this effect was mediated

by activation of AMPA/Kainate receptors (Gegelashvili et al. 1996). Another study showed that glutamate also increases the cell surface expression of GLAST on astrocytes (Duan et al. 1999).

Additionally, stimulation of different types of glutamate receptors in astrocytes could have either a positive or negative effect on the expression of the transporters. For example, activation of metabotropic glutamate receptors (mGluRs) group II upregulates GLAST mRNA and protein expression, while stimulation of group I mGluRs inhibits the expression of both GLAST and GLT-1, and both mediate their effects via activation of ERK/PI3K/ NF-κB pathway (Lin et al. 2014). On the other hand, activation of ionotropic glutamate receptors (iGluRs) suppresses GLAST expression via activation of protein kinase C (PKC) signaling pathway (Lopez-Bayghen and Ortega 2004).

Co-culturing astrocytes with neurons or neuron-conditioned medium (NCM) is a major factor that increases both GLT-1 and GLAST protein expressions and functional activities (Swanson et al. 1997). This effect could be attributed to the soluble molecules released from neurons to the culture medium such as pituitary adenylate cyclase-activating polypeptide (PACAP), whose effects on GLT-1 and GLAST were mediated by activation of PKC and protein kinase A (PKA) signaling pathways (Figiel and Engele 2000).

Various other factors mediate a positive effect on GLAST and/or GLT-1 mRNA and protein expression including hormones such as estrogen and glucocorticoids, growth factors such as epidermal growth factor (EGF), glial cell line-derived neurotrophic factor (GDNF), basic fibroblast growth factor (bFGF), and IGF-1, as well as treating astrocyte cultures with dibutyryl cyclic adenosine monophosphate (dbcAMP), ATP or adenosine. All these factors mediate their positive effects on glutamate transporters' through activation of phosphoinositide 3-kinase (PI3K), nuclear factor-κB (NF-κB) or PKA signaling pathways (Chi-Castañeda, Suárez-Pozos, and Ortega 2017; Gegelashvili and Schousboe 1997; Mahmoud et al. 2019; Sattler and Rothstein 2006).

By contrast, other factors mediate a negative effect on the expression of one or both transporters involving, insulin hormone, endothelins, dopamine, and retinoic acid, in addition to many neuropathological conditions in the CNS such as brain ischemia and inflammatory conditions associated with the release of tumor necrosis factor-α (TNF-α). The repression of the transporters' expression is achieved via the activation of PKA, NF-κB and YY1 pathways, and by suppressing JAK/STAT signaling pathway (Chi-Castañeda et al. 2017; Gegelashvili and Schousboe 1997; Mahmoud et al. 2019; Sattler and Rothstein 2006).

From the previous studies, it is obvious that depending on the interaction with environmental factors and other signaling pathways, activation of various singling pathways such as NF-κB, PKA or PKC could mediate either activation or inhibition of the expression of glutamate transporters in astrocytes (Mahmoud et al. 2019).

1.2.6.2. Post-translational modifications and regulation of the transporter activity

It is well-known that both GLAST and GLT-1 express potential sites of glycosylation. However, two independent studies revealed that glycosylation of both proteins during protein maturation does not have any effect on their protein trafficking to the cell surface or their functional activity (Conradt, Storck, and Stoffel 1995; Raunser et al. 2005).

Another study in 2004 showed that the interaction with the membrane cholesterol is essential to stabilize EAAT2 protein on the cell membrane and maintain its glutamate uptake ability (Butchbach et al. 2004).

Many contradictory reports were published regarding direct protein phosphorylation of GLAST and GLT-1 by PKC, PI3K or PKA, where different studies reported that they upregulate, have no effect or downregulate the movement of one or both transporter proteins to the astrocytic plasma membrane (Chi-Castañeda et al. 2017; Gegelashvili and Schousboe 1997; Mahmoud et al. 2019; Sattler and Rothstein 2006).

Moreover, various elements were shown to influence the glutamate uptake activity of one or both transporters. Arachidonic acid (AA) is one of these factors that is known to be a differential regulator, as it downregulates EAAT1 activity, while it upregulates glutamate uptake by EAAT2 (Zerangue et al. 1995). In patients with Alzheimer's disease (AD), Amyloid β-peptide was shown to inhibit the activity of glutamate transporters (Masliah et al. 1996), while in another study, it was shown to increase trafficking of GLAST protein to the cell membrane and enhance its functional activity (Ikegaya et al. 2002).

One of the major cellular factors that inhibit the functional activity of glutamate transporters in astrocytes is oxidative stress. Two different reports showed that oxidative stress caused by hydrogen peroxide (H_2O_2) was associated with reduced glutamate uptake in astrocyte cultures due to direct oxidation of the sulfhydryl (SH) group of both transporter proteins (Trotti et al. 1997; Volterra et al. 1994). This negative effect of H_2O_2 was eliminated by treating the cultures with superoxide dismutase and catalase anti-oxidant enzymes (Sorg et al. 1997). In a recent study on

ascorbate (an antioxidant released by astrocytes during glutamate uptake and protects against glutamate neurotoxicity), ascorbate-deficient mice showed behavioral changes and a higher susceptibility to epilepsy than wild-type (WT) mice. In the same study, the authors demonstrated that ascorbate deficiency might explain the development of subclinical seizures in patients with AD (Mi et al. 2018).

1.3. Glutamate release by astrocytes in the CNS

This section is modified from my published review (Mahmoud et al. 2019).

In addition to glutamate uptake, recent studies revealed that astrocytes release traces of glutamate to the extracellular space, which modulates neuronal firing. The interest in astrocytic glutamate release started in 1990 when Cornell-Bell and colleagues reported that glutamate release from glutamatergic neurons induced intracellular Ca^{2+} ($[Ca^{2+}]_i$) level elevation in astrocytes (Cornell-Bell et al. 1990), followed by elevation of the $[Ca^{2+}]_i$ in the adjacent neurons (Nedergaard 1994). Later on, it was shown that this high $[Ca^{2+}]_i$ level in astrocytes evoked glutamate release from astrocytes (Parpura et al. 1994).

1.3.1. Functions of astrocytic glutamate release

The released glutamate from astrocytes plays many vital roles that modulate neuronal firing and regulate the synaptic activity (Hamilton and Attwell 2010; Mahmoud et al. 2019). These roles include:

Synchronization and strengthening of the firing of excitatory neurons. The released glutamate from glutamatergic neurons stimulates type I and V mGluRs in astrocytes, which induce elevation of $[Ca^{2+}]_i$ level in astrocytes, that in turn evokes glutamate release. The released glutamate from astrocytes activates group I mGluRs or N-methyl-D-aspartate (NMDA) receptors in the adjacent neurons which increases the amount of glutamate release from the excitatory neurons and generates neuronal slow inward currents that are thought to synchronize their firings (Fellin et al. 2004; Jourdain et al. 2007).

Potentiation of neuronal inhibition. GABA release from the inhibitory neurons stimulates $GABA_B$ receptors in astrocytes, which triggers $[Ca^{2+}]_i$ rise and induces glutamate release from astrocytes. Through activation of neuronal presynaptic iGluRs, the released glutamate from astrocytes increases GABA release from the adjacent inhibitory neurons (Kang et al. 1998).

Potentiation of the transient hetero-synaptic inhibition in the hippocampus. The released glutamate from the excitatory afferents to CA1 pyramidal cells stimulates interneurons to release GABA. GABA activates $GABA_B$ receptors in astrocytes, which elevates $[Ca^{2+}]_i$ and triggers glutamate release from astrocytes. This glutamate acts on presynaptic Group I and III mGluRs in the adjacent afferents to inhibit glutamate release (Andersson, Blomstrand, and Hanse 2007).

1.3.2. Mechanisms of astrocytic glutamate release

Glutamate release by astrocytes is mediated primarily by Ca^{2+}-dependent exocytosis; however, several other Ca^{2+}-dependent and Ca^{2+}-independent mechanisms were shown to be implicated in astrocytic glutamate release in healthy CNS and pathological conditions (Mahmoud et al. 2019; Malarkey and Parpura 2008). In this context, we summarize these mechanisms and their contribution to glutamate release from astrocytes **(Figure 4)** (Mahmoud et al. 2019).

1.3.2.1. Ca^{2+}-mediated glutamate exocytosis

Ca^{2+}-mediated exocytosis is yet considered the principal mechanism responsible for glutamate release by astrocytes in the healthy CNS **(Figure 4A)**. Similar to neurons, astrocytes express all the machinery necessary for glutamate exocytosis. In neurons, glutamate is packed inside small intracellular vesicles, from which glutamate is released to the synaptic cleft. Electron microscopic visualization of the hippocampal astrocytes revealed the presence of small intracellular vesicles (~ 30 nm), like those in neurons (Bezzi et al. 2004). Another imaging study demonstrated that rat hippocampal astrocytes contain large intracellular vesicles (~ 300 nm) containing glutamate, from which only 10% of their glutamate content is released in a “kiss and run” mechanism (Chen et al. 2005).

In addition, the small intracellular vesicles in astrocytes express the vesicular glutamate transporters 1 and 2 (VGLUT1 and VGLUT2), like those expressed by neurons. These transporters are localized in astrocytes in contact with presynaptic neurons expressing NMDA receptors. The function of these transporters is to transfer glutamate from the cytosol to the vesicles to be ready for exocytosis. The function of both transporters in astrocytes, as well as in neurons is derived by the voltage and proton gradient generated by the vacuolar (H+) ATPase across the vesicular membrane (Montana et al. 2004).

Glutamate exocytosis from neurons occurs in response to $[Ca^{2+}]_i$ elevation caused by extracellular Ca^{2+} entry via the voltage-gated Ca^{2+} channels. This rise in the $[Ca^{2+}]_i$ is sensed by the Ca^{2+} sensor, synaptotagmin 1, expressed by neurons. Unlike neurons, the main source of $[Ca^{2+}]_i$ elevation in astrocytes comes from the intracellular Ca^{2+} stores. Stimulation of the G protein-coupled receptors (GPCRs) in astrocytes activates inositol-1,4,5-trisphosphate (IP3), which in turn triggers Ca^{2+} release from the endoplasmic reticulum (ER) (Hua et al. 2004). Mitochondrial Ca^{2+} also plays a role in astrocytic glutamate release by modulating cytoplasmic Ca^{2+} dynamics (Reyes and Parpura 2008). By contrast, extracellular Ca^{2+} plays a minor role in astrocytic glutamate exocytosis (Hua et al. 2004). Further studies demonstrated that astrocytes express synaptotagmin 4, 7 or 11 (analogs of synaptotagmin 1 in neurons) (Wang et al. 2003). To trigger glutamate exocytosis from astrocytes, the $[Ca^{2+}]_i$ level should reach approximately 250–350 nM (Parpura and Haydon 2000).

To complete the process of exocytosis in neurons, in response to the $[Ca^{2+}]_i$ elevation, the vesicle fusion protein, vesicle-associated membrane protein 2 (VAMP2) attaches to the cell membrane fusion proteins, Syntaxin and synaptosomal-associated protein 25 (SNAP25) to form soluble N-ethylmaleimide-sensitive factor attachment protein receptors complex (SNARE complex) (Jahn and Scheller 2006). Similar fusion proteins are expressed in astrocytes to achieve glutamate exocytosis. Astrocytes express the vesicle fusion protein VAMP2 or its analog VAMP3 (Bezzi et al. 2004), and the cell membrane fusion proteins, Syntaxin and SNAP23 (analog of SNAP25 in neurons) (Hepp et al. 1999; Parpura et al. 1995). Therefore, they have the ability to form a SNARE complex, as do neurons, resulting in glutamate release from the vesicles to the extracellular space.

1.3.2.2. Bestrophin-1 and TREK-1 channel-mediated glutamate release

A newly identified Ca^{2+}-mediated glutamate release channel is called Bestrophin-1 (Best-1 channel) **(Figure 4B)**. Glutamate release through Best-1 channels also occurs in response to GPCR stimulation in astrocytes, which triggers a rise in the $[Ca^{2+}]_i$ that induces glutamate release. However, it is not yet identified whether these channels can directly sense the rise in $[Ca^{2+}]_i$ or through a Ca^{2+} sensor. The released glutamate via Best-1 channels targets the synaptic NMDA receptors in neurons and generates inward neuronal currents that are much slower than those generated by glutamate exocytosis. TREK-1 channels are other newly identified channels that

release glutamate in a Ca^{2+}-independent mechanism **(Figure 4C)**. They are K^+ channels that allow the outward transport of glutamate in exchange with K^+. The released glutamate via TREK-1 channels targets mGluRs in neurons and generates neuronal currents that are very rapid compared to those generated by Best-1-mediated glutamate release (Han et al. 2013; Woo et al. 2012).

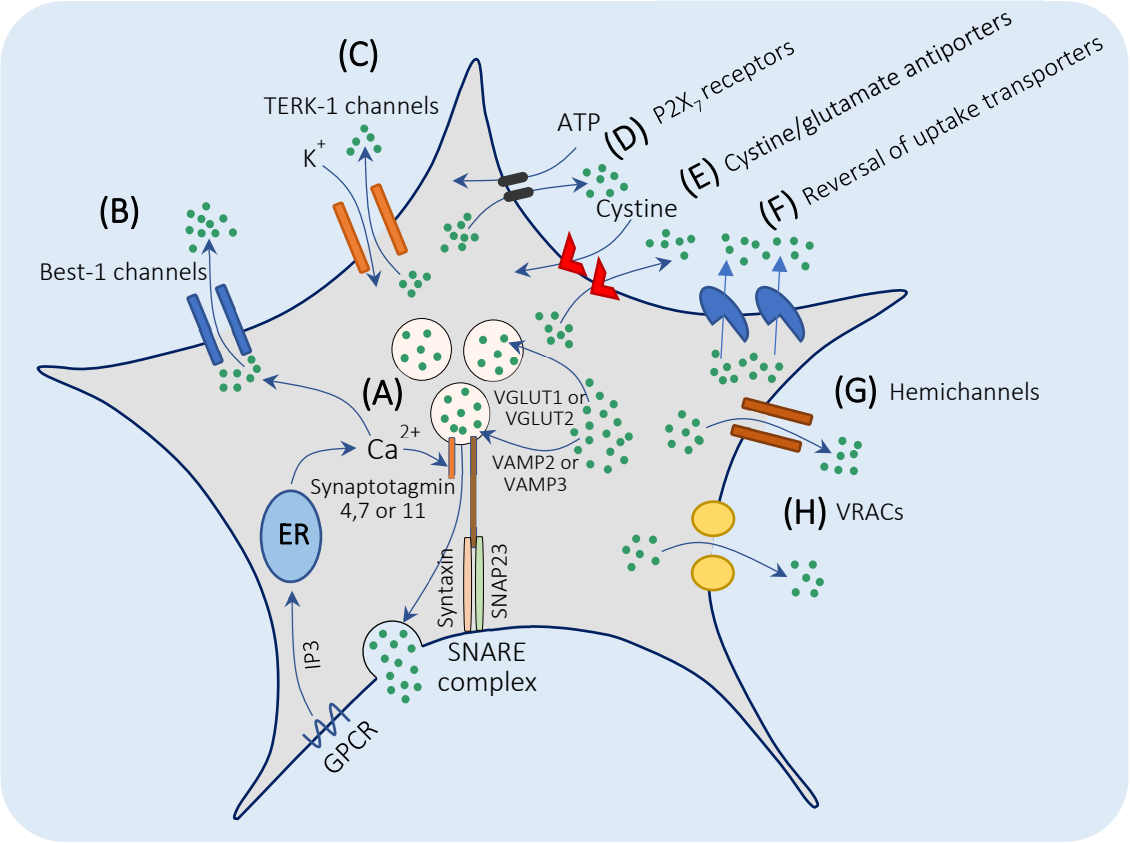

Figure 4: Mechanisms of glutamate release by astrocytes. (A) <u>Ca^{2+}-mediated exocytosis</u>: astrocytes express VGLUT1 and VGLUT2, which transport glutamate to the intracellular vesicles. Stimulation of the GPCRs in astrocytes results in Ca^{2+} release from the ER in response to IP3 formation. Sensing the intracellular Ca^{2+} elevation by synaptotagmin 4, 7 or 11, induces fusion of the vesicular protein VAMP2 or VAMP3 with the cell membrane fusion proteins Syntaxin and SNAP23 to form SNARE complex, resulting in glutamate release. **(B)** <u>Best-1 channels:</u> glutamate release occurs in a Ca^{2+}-dependent mechanism following GPCR stimulation. **(C)** <u>TREK-1 channels:</u> glutamate is released in exchange with K^+ uptake. **(D)** <u>$P2X_7$ receptors:</u> glutamate release occurs in exchange with ATP uptake. **(E)** <u>Cystine/glutamate antiporters:</u> glutamate is released in exchange with cystine uptake. **(F)** <u>Reversal of uptake transporters:</u> in severe ischemia or stroke. **(G)** <u>Gap junction hemichannels:</u> formed by connexin and pannexin proteins. **(H)** <u>Volume-regulated anion channels (VRACs):</u> as in the case of brain edema (Mahmoud et al. 2019).

1.3.2.3. Glutamate release through $P2X_7$ receptors

P2X Purinoceptor 7 ($P2X_7$) receptors are ATP uptake receptors. When these receptors are activated in astrocytes *in vitro*, they allow the release of glutamate in exchange with the inward transport of ATP (Duan et al. 2003) **(Figure 4D)**. However, the contribution of these receptors to the glutamate release *in vivo* is still unclear.

1.3.2.4. Cystine/glutamate antiporters

They are Cl^- dependent cystine uptake transporters that allow the outward transport of glutamate in exchange with the cystine uptake **(Figure 4E)**. In two different studies, activating these transporters in the cerebellum and rat striatum induced glutamate release from astrocytes and generated inward currents in the adjacent neurons (Baker et al. 2002; Warr, Takahashi, and Attwell 1999). In a third study, adding cystine to the acutely-cut brain slices increased the level of the extracellular glutamate (Moran et al. 2005).

1.3.2.5. Reversal of glutamate uptake transporters

This mechanism of glutamate release occurs only in pathological conditions. In case of reversal of the driving forces of glutamate uptake such as reversal of intracellular and extracellular Na^+/K^+ ratio or ATP depletion as in case of severe ischemia, glutamate transporters lose their function of glutamate uptake, and they may reverse, resulting in glutamate release via a Ca^{2+}-independent mechanism (Grewer et al. 2008; Phillis and O'Regan 1996; Rossi, Oshima, and Attwell 2000) **(Figure 4F)**.

1.3.2.6. Gap junction hemichannels

The gap junctions connecting astrocytes are formed by Connexin and Pannexin proteins arranged in the form of hemichannels. Activation of these hemichannels *in vitro* in ischemic astrocytes results in the release of glutamate from the cytoplasm to the extracellular space in a Ca^{2+}-independent mechanism (Ye et al. 2003) **(Figure 4G)**.

1.3.2.7. Volume-regulated anion channels (VRACs)

Glutamate release through the VRACs was reported to occur in pathological conditions, *in vitro* in swollen astrocytes, and *in vivo* in brain edema and stroke. In these conditions, VRACs

open and allow the outward transport of glutamate (Kimelberg et al. 1990; Seki et al. 1999) **(Figure 4H)**. However, it is not yet known whether this mechanism of glutamate release is Ca^{2+}-dependent or independent (O'Connor and Kimelberg 1993).

1.4. Glutamate excitotoxicity

This section is modified from my published review (Mahmoud et al. 2019).

Under physiological conditions, glutamate concentration in the extracellular space of the CNS must be maintained at very low levels to prevent overstimulation of glutamate receptors in neurons and its subsequent neuronal death.

Under pathological conditions, the pathogenesis of almost all CNS disorders is associated with inflammation. Astrocytes respond to inflammation by undergoing molecular, morphological, and functional changes in a process known as "reactive astrogliosis". Reactive astrocytes may lose some of their vital functions in the CNS or acquire many harmful effects that exacerbate the inflammatory response and delay the process of recovery. Reactive astrogliosis may be associated with loss of the astrocytic function of glutamate uptake and/or excessive glutamate release, which lead to accumulation of glutamate in the extracellular space and predispose to glutamate excitotoxicity (Sofroniew and Vinters 2010).

Glutamate excitotoxicity is defined as "the process by which neuronal death, by apoptosis or necrosis, occurs as a result of excessive or prolonged exposure of neurons to the extracellular glutamate" (Mahmoud et al. 2019).

1.4.1. CNS disorders associated with glutamate excitotoxicity

Glutamate excitotoxicity caused by reduced astrocytic glutamate uptake occurs in many CNS diseases such as CNS trauma. The expression of both glutamate transporters, EAAT1 and EAAT2, is significantly reduced in astrocytes for up to 7 days post-trauma (Landeghem et al. 2006). The same effect on the transporters occurs following CNS infection with human immunodeficiency virus (HIV) (Vesce et al. 1997).

Neurodegenerative diseases are commonly associated with impaired glutamate uptake. In amyotrophic lateral sclerosis (ALS), the affected patients suffer from loss of functions of the motor neurons caused by loss of EAAT2 expression in the spinal cord astrocytes (Rothstein et al. 1995). Interestingly, β-lactam antibiotics were neuroprotective in the animal models of ALS, as β-lactam

upregulates the expression and the functional activity of GLT-1 (EAAT2) in the CNS of these animal models (Rothstein et al. 2005). Inhibition of glutamate uptake may also be involved in other neurodegenerative diseases such as Parkinson's disease (PD), AD, Huntington's disease and epilepsy (Sheldon and Robinson 2007).

Many *in vitro* and *in vivo* studies demonstrated that glutamate excitotoxicity is implicated in the pathogenesis of multiple sclerosis (MS), an autoimmune disease affecting the CNS. Incubating astrocytes *in vitro* with MOG-activated T-cells (myelin-specific T-cells) induced marked reduction of GLAST expression in astrocytes, and this effect was mediated by T-cell-released TNF-α (Korn, Magnus, and Jung 2005). *In vivo* studies on experimental autoimmune encephalomyelitis (EAE, a mouse model of MS) revealed that reduced glutamate uptake in the CNS resulted in the death of neurons and oligodendrocytes, and exacerbated the process of demyelination (Pitt, Werner, and Raine 2000). Moreover, several studies on human MS revealed that glutamate excitotoxicity is involved in the pathogenesis of the disease (Werner, Pitt, and Raine 2001). Therefore, riluzole, an anti-glutamatergic drug, is now in clinical trials for the treatment of early onset MS (onset of less than one year) (Waubant et al. 2014).

Total failure of glutamate uptake occurs in CNS disorders associated with reversal or depletion of the driving forces of glutamate uptake such as the depletion of intracellular ATP that may occur in brain ischemia or stroke (Grewer et al. 2008; Phillis and O'Regan 1996; Rossi et al. 2000). Reversal of the Na^+/K^+ ratio is another example that occurs in hepatic encephalopathy, in which intracellular Na^+ is markedly augmented as a result of ammonium toxicity (Kelly et al. 2009). In addition to the glutamate uptake failure in these diseases, reversal of glutamate uptake transporters also may occur, as previously described, resulting in glutamate release, which exacerbates the glutamate excitotoxicity (Grewer et al. 2008; Phillis and O'Regan 1996; Rossi et al. 2000).

Glutamate excitotoxicity caused by abnormal excessive release of glutamate from astrocytes commonly occurs in CNS disorders accompanied by increased release of the inflammatory mediators, TNF-α and prostaglandin E (Bezzi et al. 1998, 2001). These two mediators trigger a rise in the $[Ca^{2+}]_i$ level in astrocytes, which in turn enhances glutamate exocytosis, as occurs in the CNS infection with HIV, brain stroke, AD and MS (Hamilton and Attwell 2010).

Various studies on neuro-psychiatric disorders demonstrated that combined dysregulation of both glutamate uptake and release by astrocytes contributes to the development of mood disorders and depression-like symptoms in animal models. Also, it was shown to be implicated in the

pathogenesis of the major depressive disorder (MDD) and schizophrenia in human (Bechtholt-Gompf et al. 2010; Haroon, Miller, and Sanacora 2017; Takahashi, Foster, and Lin 2015). Therefore, riluzole (the anti-glutamatergic drug) is used as an anti-depressant treatment for patients with MDD (Sanacora et al. 2007; Zarate et al. 2004).

1.4.2. Molecular mechanisms of glutamate excitotoxicity

Hyperstimulation of neuronal glutamate receptors results in the activation of several molecular pathways that interact synergistically and lead eventually to neuronal death. Here, we summarize the key molecular mechanisms involved in the pathogenesis of glutamate excitotoxicity **(Figure 5)** (Mahmoud et al. 2019).

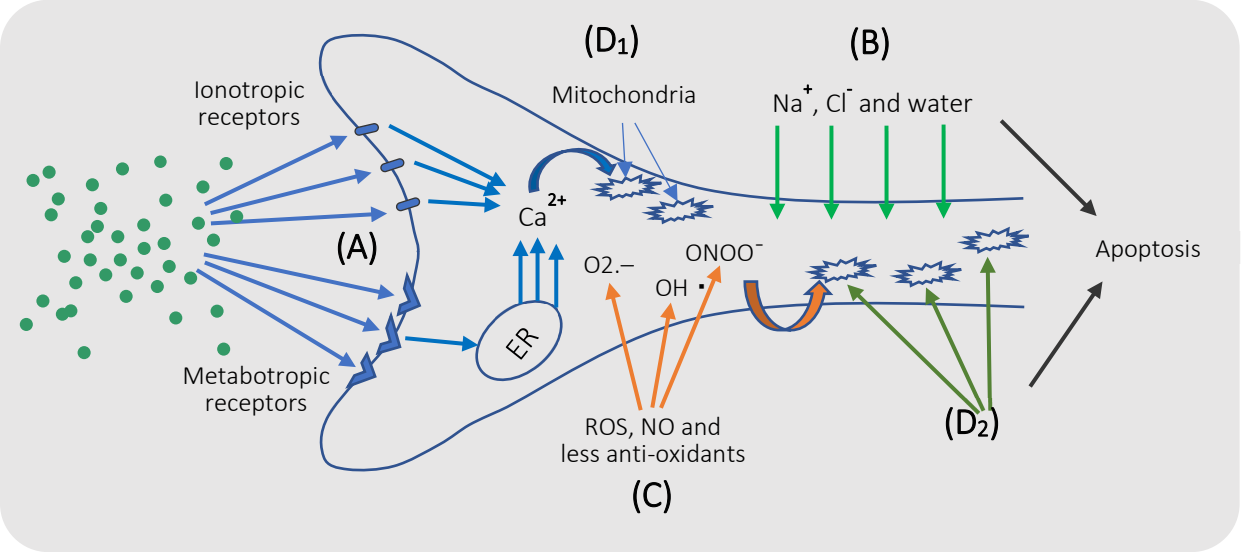

Figure 5: Molecular mechanisms of glutamate excitotoxicity. (A) Excess extracellular glutamate triggers hyperexcitation of neuronal ionotropic glutamate receptors and metabotropic receptors, resulting in augmentation of the $[Ca^{2+}]_i$ level caused by excess Ca^{2+} entry via ionic channels and excess Ca^{2+} release from the ER, respectively. This elevation in the $[Ca^{2+}]_i$ level represents a signal for apoptosis. **(B)** The rapid influx of Na^+ and Cl^- ions, and water to neurons results in rupture of the neuronal plasma membrane. **(C)** Oxidative stress caused by excess ROS, RNS, and the deficiency of antioxidants induces damage to the cellular proteins and nucleic acid, which in turn triggers apoptosis. **(D)** Excessive Ca^{2+} entry into the mitochondria **(D1)** and mitochondrial overstimulation by oxidative stress **(D2)** result in the opening of mitochondrial transition pores and release of Cytochrome C which initiate the apoptotic cascades (Mahmoud et al. 2019).

First, excess glutamate in the extracellular space, caused by reduced glutamate uptake or increased glutamate release by astrocytes induces hyperstimulation of glutamate ionotropic receptors in neurons, which are NMDA, α-amino-3-hydroxy-5-methylisoxazole-4-propionate

(AMPA), and kainic acid (KA) receptors. The stimulation of which triggers Ca^{2+} entry through the voltage-gated anion channels in neurons. Excess glutamate also hyperstimulates group I and V mGluRs in neurons, which in turn triggers the formation of IP3 and Ca^{2+} release from the ER. The resulting high $[Ca^{2+}]_i$ level will raise the Ca^{2+} concentration in the Ca^{2+}-sensitive organelles, mitochondria and ER, which represents the first signal to induce neuronal apoptosis. Also, the elevated $[Ca^{2+}]_i$ level activates apoptotic proteases such as calcineurin and calpain to induce apoptosis (Dong, Wang, and Qin 2009; Mahmoud et al. 2019; Wang and Qin 2010).

Second, the excessive Ca^{2+} entry mediated by hyperstimulation of NMDA receptors causes excessive influx of water and ions such as Na^+ and Cl^- into neurons, which in turn induces acute rupture of the neuronal plasma membrane and neuronal death by necrosis (Dong et al. 2009; Mahmoud et al. 2019; Wang and Qin 2010).

Third, the pathogenesis of glutamate excitotoxicity is often associated with oxidative stress, caused by excessive production of reactive oxygen species (ROS), especially superoxide (O_2^-) and hydroxyl radicals (OH.). Neurodegenerative diseases also could be associated with reduced levels of antioxidants. Excessive reactive nitrogen species (RNS) also play a role in oxidative stress. Excess Ca^{2+} entry via the cation channels mediated by NMDA receptor stimulation activates nitric oxide synthetase (NOS) enzyme that synthesizes NO. NO interacts with O_2^- to produce peroxynitrite ($OONO^-$). The resulting oxidative stress causes damage to the intracellular proteins, nucleic acid, lipids, and other molecules, resulting in activation of the intracellular apoptotic pathways (Dong et al. 2009; Mahmoud et al. 2019; Wang and Qin 2010).

Finally, Excessive mitochondrial stimulation by the high $[Ca^{2+}]_i$ levels and oxidative stress results in the opening of mitochondrial permeability transition pores and release of pro-apoptotic molecules into the cytosol such as cytochrome C, which activate the mitochondria-mediated apoptotic pathways (Dong et al. 2009; Mahmoud et al. 2019; Wang and Qin 2010).

1.5. Pattern recognition receptors (PRRs)

PRRs are groups of receptors expressed mainly by innate immune cells. They detect unique molecular structures of conserved pathogen-associated molecular patterns (PAMPs) in the structure of the microorganisms that are important for their physiology such as the cell membrane and the nuclei. They can also recognize patterns from the released molecules of tissue damage or stress, known as damage-associated molecular patterns (DAMPs) such as heat shock proteins

(HSP), damaged nucleic acid, and ATP. Upon recognition of PAMPs or DAMPs, PRRs initialize series of signaling cascades that lead to the activation of transcription factors such as NF-κB and MAPK, and production of chemokines and cytokines that recruit and activate other innate and adaptive immune cells (Mogensen 2009). PRRs are divided into 4 groups of receptors which are situated either on the cell membrane or intracellularly. These groups are:

1.5.1. Toll-like receptors (TLRs)

TLRs are a big group of PRRs that is expressed in most of the cell types. 10 subtypes of TLRs were identified, among which TLRs 1, 2, 4, 5, 6, and 10 are transmembranous and can recognize extracellular molecules such as bacterial lipopolysaccharide (LPS) and flagellin. Once the surface TLRs become activated, they activate NF-κB transcription factor, that leads to the production of pro-inflammatory mediators including chemokines, cytokines, and ROS that activate phagocytosis, recruit more neutrophils and macrophages to the site of infection, activate cells of the adaptive immune response, and accelerate clearance of the microorganism. TLRs 3, 7, 8, and 9 are expressed in the endosomes and can recognize virus single-stranded RNA (ssRNA) or double-stranded RNA (dsRNA), and unmethylated CpG DNA. When endosomal TLRs get activated, they activate interferon regulatory factors (IRFs), which induce the production of type I interferon (IFN-α and IFN-β) to eradicate the viral infection (Dowling and Mansell 2016).

1.5.2. C-type lectin receptors (CLRs)

This group of PRRs is expressed on the cell surface and can recognize carbohydrate structures of the microorganism such as mannose, fucose, and glucan. Recognition of these molecules results in internalization and phagocytosis of the pathogen followed by antigen presentation. Therefore, CLRs are expressed mainly in the antigen-presenting cells (APCs) such as monocytes, macrophages, and dendritic cells (DCs). Furthermore, activation of the mannose-binding lectins activates the complement system, which can fight the infection in several ways; direct lysis of the pathogen, generation of anaphylatoxins, and opsonization of the infected cells (Dunkelberger and Song 2010).

1.5.3. RIG-I-like receptors (RLRs)

RLRs are intracellular PRRs expressed in the cytosol of immune and non-immune cells. 3 members of this group were identified, which are RIG-1, melanoma differentiation associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2). They recognize viral RNA, and upon activation, RLRs activate the antiviral signaling pathways to produce type I interferons (Sparrer and Gack 2015).

1.5.4. NOD-like receptors (NLRs)

They are also known as the nucleotide-binding and leucine-rich repeat-containing proteins (NLRs). NLRs are expressed in the cytoplasm of immune cells such as macrophages, dendritic cells, and neutrophils, as well as non-immune cells. They can sense a wide variety of PAMPs such as bacterial peptidoglycans and viral RNA, in addition to DAMPs from uric acid, ATP, nucleic acid proteins, ROS, alterations in osmolarity, and polysaccharides (Kumar, Kawai, and Akira 2011). The importance of NLRs in the host defense is highlighted by being evolutionary conserved across different species including plants and mammals (Jha and Pan-Yun Ting 2015). Also, genetic mutations in several members of NLRs are associated with the development of many human diseases such as Cryopyrin-Associated Periodic Syndromes (CAPS), which are auto-immune syndromes associated with a mutation in *Nlrp3* gene (Lequerre et al. 2006). Mutations in *Nlrp1* were shown to be associated with vitiligo (Jin et al. 2007), and mutations in the *NOD2* gene is associated with Blau Syndrome (Miceli-Richard et al. 2001).

According to their structure, NLR family members contain 3 characteristic domains **(Figure 6A)**, the C-terminal leucine-rich repeat (LRR), which is the ligand binding domain, the central nucleotide binding domain (NBD), which is responsible for oligomerization of the protein, and the N-terminal domain, which is the effector domain (Allen 2014; Gharagozloo et al. 2018). Till now, 23 members of the NLR family were identified in human, and more than 34 were identified in mice (Franchi et al. 2009). According to the difference in their N-terminal domain structure, NLRs are classified into 4 sub-categories **(Figure 6B)**, (1) NLRA, including only one family member, Class II transactivator (CIITA), in which the effector domain is the acidic transactivation (AD) domain, (2) NLRB, possesses baculovirus inhibitor of apoptosis protein repeat (BIR), (3) NLRC,

contains caspase recruitment domain (CARD) or unknown (X) domain, and (4) NLRP, containing pyrin domain (PYD) (Barbé, Douglas, and Saleh 2014; Gharagozloo et al. 2018).

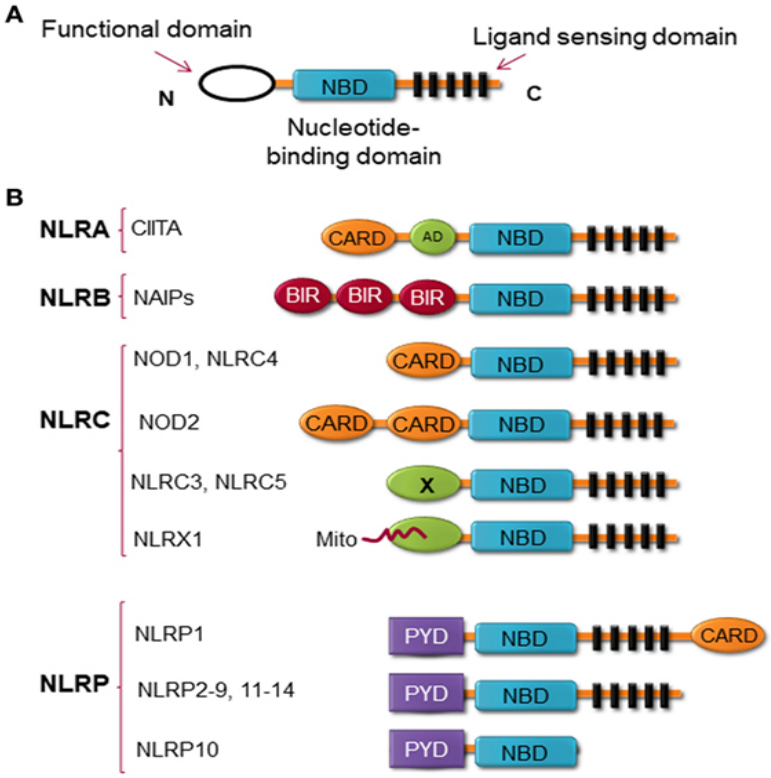

Figure 6: Structure of NLRs. (A) NLR proteins contain 3 domains, the C-terminal LRR, which is the ligand sensing domain, the central NBD, and the N-terminal functional domain. **(B)** According to the structure of the functional domain, NLRs contain 4 subgroups: NLRA with an AD domain, NLRB with a BIR domain, NLRC with a CARD or X (unknown) domains. One member of the NLRC group, NLRX1, contains a Mito sequence, which is the mitochondrial addressing sequence that localizes the protein to the mitochondria, and NLRP with a PYD domain (Gharagozloo et al. 2018).

According to their functions, NLRs are classified into 2 main categories, the inflammasome and the non-inflammasome NLRs **(Figure 7)**. The inflammasome NLRs are proteins that can assemble into large cytoplasmic multiprotein complexes such as NLRP1, NLRP3, NLRC4, NLRC5, and NLRP12. They cleave pro-caspase 1 to active caspase-1, which in turn cleaves pro-IL-1β and pro-IL-18 into the active pro-inflammatory cytokines IL-1β and IL-18. On the other side, the non-inflammasome NLRs contains 2 further subgroups. The first one contains the transcriptional co-activators, CIITA and NLRC5, which are the master regulators of MHC-II and MHC-I expressions, respectively. The second subgroup contains the regulators of NF-κB

activation, which involve those proteins that activate NF-κB, such as NOD1 and NOD2, and result in the production of pro-inflammatory mediators, and other members that inhibit NF-κB such as NLRX1 and NLRP12, thus suppressing the inflammatory response. It is noteworthy to say that 2 NLR family members, NLRP12 and NLRC5, could have either inflammasome or non-inflammasome functions in different contexts according to the cell type and the stimulator of inflammation (Gharagozloo et al. 2018).

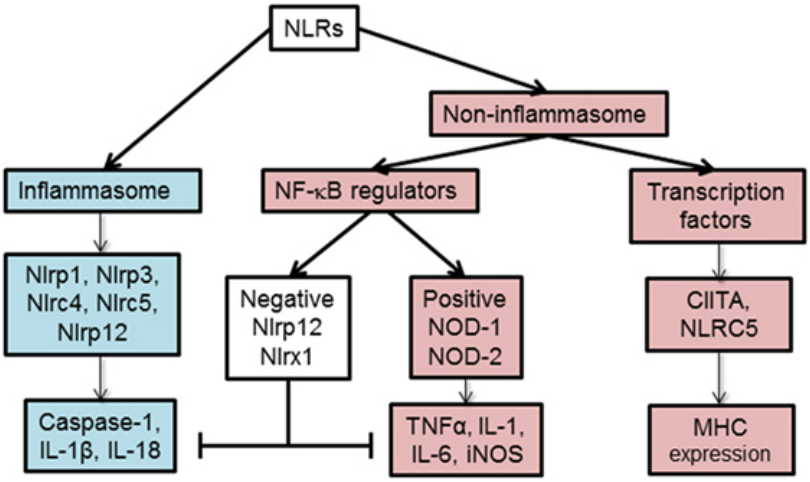

Figure 7: Functional classification of NLRs. Functionally, NLRs could be categorized into the inflammasome or non-inflammasome forming members. The inflammasome proteins are those that activate caspase-1 and result in the release of IL-1β and IL-18. The non-inflammasome members could be either transcription factors that regulate the expression of MHC-I and MHC-II, or the regulators of NF-κB, which are either positive or negative regulators of inflammation (Gharagozloo et al. 2018).

1.5.4.1. NOD-like receptor X1 (NLRX1)

NLRX1 is a recently discovered member of the non-inflammasome NLRs that inhibit NF-κB. It is ubiquitously expressed, and unlike other members of NLRs, NLRX1 possesses a mitochondrial addressing sequence in its N-terminal domain that localizes the protein to the mitochondria (Moore et al. 2008). However, its exact location in the mitochondria is still unclear. A study in 2008 demonstrated that NLRX1 is located in the outer mitochondrial membrane (Moore et al. 2008) while 3 other more recent studies showed that NLRX1 is situated in the mitochondrial matrix (Arnoult et al. 2009; Rhee et al. 2013; Singh et al. 2018).

Regarding the role of NLRX1 in the regulation of anti-microbial immune response, many contradictory studies were published using different models of infection. Early in 2008, Tattoli et al. reported that NLRX1 enhanced ROS production in the cells treated with TNF-α, *shigella*

infection and dsRNA, and this high level of ROS activated NF-κB and JUN amino-terminal kinases-dependent (JNK) signaling pathways (Tattoli et al. 2008). By contrast, several subsequent studies provided evidence that NLRX1 is a negative regulator for NF-κB. After treatment with LPS *in vivo* and *in vitro*, NLRX1 was shown to inhibit TLR-mediated activation of NF-κB, by interacting with TRAF6 in its canonical pathway (Allen et al. 2011; Xia et al. 2011), and by inhibiting phosphorylation of IKKα and IKKβ, which results in the suppression of NF-κB pathway (Xia et al. 2011).

During viral infections, viral PAMPs are recognized by RIG-1 protein in the cytoplasm, which in turn activates the mitochondrial antiviral signaling (MAVS) pathway. The activation of which, in turn, activates the interferon regulatory factors (IRFs) that induce the production of type I interferons to eliminate the viral infection. In 2 studies done by Moore et al. and Allen et al., NLRX1 was shown to interact with MAVS adaptor in the outer mitochondrial membrane and inhibits RIG-1-MAVS signaling pathway, which inhibits the production of IFN type I (Allen et al. 2011; Moore et al. 2008). In a recent study in 2019, Song et al. demonstrated that the central NBD (NACHT) of NLRX1 protein is necessary for its interaction with MAVS to suppress the antiviral immune response (Song et al. 2019).

In another study, Lei et al. showed that NLRX1 interacts with Tu translation elongation factor (TUFM) in the mitochondrial matrix, which promotes autophagy during viral infection; therefore, it inhibits type I IFN production (Lei et al. 2012). Interestingly, another group confirmed the role of NLRX1 in promoting autophagy in macrophages by the same mechanism in a model of fungal infection by *Histoplasma capsulatum* (Huang et al. 2018). However, in a recent study, NLRX1 was reported to suppress the invasion and autophagy of group A streptococcal infection, by interacting with Beclin-1 and UVRAG members of Beclin-1 complex, that eventually inhibits autophagosome and autolysosome formation (Aikawa et al. 2018). From the previous reports, it is clear that NLRX1 can mediate a positive or negative effect on the anti-microbial immune response depending on the experimental conditions and the nature of the microorganism.

Regarding sterile inflammation, NLRX1 was shown to inhibit inflammatory bowel disease (IBD) in mice and decrease its associated colonic lesions and inflammatory mediators (Leber et al. 2018). NLRX1 also protects against colitis induced by dextran sodium sulfate (Leber et al. 2017) and inhibits colitis-associated cancer in mice by suppressing NF-κB pathway (Tattoli et al.

2016). In an *in vitro* model of osteoarthritis, Ma et al. reported that NLRX1 overexpression inhibits LPS-induced apoptosis and inflammation in chondrocytes also by repressing NF-κB (Ma et al. 2019).

In the CNS, Eitas et al. showed that NLRX1 protects against the development of EAE (a mouse model of MS). CNS tissue staining showed more tissue damage with enhanced demyelination in the spinal cord of *Nlrx1* knockout (*Nlrx1*$^{-/-}$) mice relative to WT. Flow cytometry analysis indicated that there was a higher infiltration of macrophages/microglia and T-cells into the spinal cord tissue of *Nlrx1*$^{-/-}$ mice, associated with increased mRNA expression of pro-inflammatory cytokines *Tgfb-1*, *Ifn-ɣ*, *Il-17a*, *Il-17f*, and *Il-22*. *Nlrx1*$^{-/-}$ mice also showed enhanced microglial activation and higher inflammatory response than WT mice, evidenced by increased expression of MCH-II and increased production of the pro-inflammatory mediators, IL-6 and CCL2 (Eitas et al. 2014).

In a mouse model of traumatic brain injury, *Nlrx1*$^{-/-}$ mice had larger brain lesions and more motor neurological deficits following a controlled cortical impact (CCI) injury compared to WT mice. At the cellular level, *Nlrx1*$^{-/-}$ mice were associated with higher proliferation and infiltration of microglia at the lesion sites. NF-κB signaling was also upregulated in the microglia of *Nlrx1*$^{-/-}$ mice compared to WT. NLRX1 also protected neurons from cell death following oxidative stress *in vitro* (Theus et al. 2017). These data suggest that NLRX1 negatively regulates sterile inflammation by inhibiting NF-κB activation.

Concerning the role of NLRX1 in human diseases, there is only one published study linking a mutation in *nlrx1* to chronic hepatitis B (CHB) virus infection. The authors discovered a missense mutation in the LRR of *nlrx1* associated with increased susceptibility to CHB infection in human patients, based on a study involved 50 CHB patients versus 40 controls (Zhao et al. 2012).

Additionally, the level of the mRNA or protein expression of NLRX1 is altered in various human diseases. In a study published in 2015, by Kang and colleges, the authors revealed that the mRNA expression of *nlrx1* is significantly reduced in the lung tissues of patients with chronic obstructive pulmonary disease (COPD) relative to controls. This *nlrx1* suppression positively correlated with the disease severity and negatively correlated with the pulmonary function (Kang et al. 2015). In another study in 2017, Stokman et al. examined NLRX1 protein expression using IHC, in renal biopsies from patients with acute tubular necrosis (ATN) and acute cellular rejection (ACR). NLRX1 was also significantly reduced in both ATN and ACR compared to healthy

controls (Stokman et al. 2017). In the CNS, there is only one publication evaluated the gene expression of *nlrx1* by microarray meta-analysis in patients following ruptured brain aneurysms. In these patients, *nlrx1* expression was markedly downregulated relative to controls (Theus et al. 2017).

Due to its localization to the mitochondria, NLRX1 regulates mitochondrial dynamics and functional activities. In a previous publication from our group, we used NLRX1 Knock-In (KI) and Knock-Down (KD) N2A neuroblastoma cell lines, and cells transfected with scrambled shRNA (Sc) as controls. We showed that NLRX1 inhibits neuronal death and shifts neuronal death from necrosis to apoptosis following rotenone treatment. Also, we showed that NLRX1 enhances mitochondrial fission in neurons by interacting with the fission protein, DRP1 (Imbeault et al. 2014). In another study, NLRX1 inhibited apoptosis of macrophages following influenza A virus infection (Jaworska et al. 2014). However, in case of cell stress or glucose deprivation, NLRX1 enhances the intrinsic apoptotic pathway (Soares et al. 2014). Moreover, NLRX1 inhibits oxidative stress and apoptosis while it enhances mitochondrial respiration and ATP production in renal tubular epithelial cells in a model of ischemia-reperfusion injury (Stokman et al. 2017), and in human breast cancer tissue (Singh et al. 2019). Taken together, these reports suggest that NLRX1 enhances mitochondrial fission, respiration, and ATP production. On the other hand, it inhibits oxidative stress and cell death and directs cell death towards apoptosis instead of necrosis in case of cellular stress.

2. HYPOTHESIS AND OBJECTIVES

We hypothesized that NLRX1 enhances glutamate uptake and inhibits glutamate release by astrocytes via enhancing mitochondrial functions.

To test our hypothesis, we investigated the role of NLRX1 in:

1- Glutamate uptake by astrocytes

2- Glutamate release by astrocytes

3- Mitochondrial functions in astrocytes including:

- ATP production
- Oxidative stress
- Ca^{2+} homeostasis

3. ARTICLE

NLRX1 Enhances Glutamate Uptake and Inhibits Glutamate Release by Astrocytes

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Shaimaa Mahmoud, Dr. Gris, and Dr. Amrani designed the study and the experiments. Shaimaa Mahmoud performed all the lab work and the statistical analysis, except figure 5B, which was done by Camille Simard. Marjan Gharagozloo participated in the flow cytometry experiments and their analysis. Marjan Gharagozloo and Camille Simard helped with animal care, genotyping, and culture preparation. Shaimaa Mahmoud wrote the first version of the manuscript. Dr. Gris and Gr. Amrani contributed to the conceptual reading and critical editing of the manuscript.

Résumé

L'absorption de glutamate provenant de l'espace extracellulaire et la libération de glutamate pour les neurones sont deux importants processus pris en charge par les astrocytes du système nerveux central (SNC). L'absorption du glutamate protège contre l'excitotoxicité causée par le glutamate tandis que la libération du glutamate pour les neurones renforce la décharge neuronale. En cas d'inflammation du SNC, les astrocytes peuvent perdre l'une de ces fonctions, voire les deux, ce qui entraîne une accumulation du glutamate extracellulaire. Cette accumulation conduit à une mort neuronale excitotoxique ce qui aggrave à son tour l'inflammation du SNC. NLRX1 est un récepteur immunitaire inné de type NOD qui inhibe les principales voies inflammatoires. Ce récepteur est localisé dans les mitochondries. Plusieurs études ont démontré que NLRX1 inhibe la mort cellulaire, augmente la production d'ATP et atténue le stress oxydatif. Dans ce projet de recherche, nous démontrons que NLRX1 potentialise l'absorption de glutamate astrocytaire en renforçant les fonctions mitochondriales et l'activité fonctionnelle des transporteurs de glutamate, en utilisant des cultures d'astrocytes primaires provenant de souris WT et *Nlrx1*$^{-/-}$. De plus, nous démontrons que NLRX1 inhibe la libération de glutamate par les astrocytes en réprimant l'exocytose de glutamate médiée par le Ca^{2+}. Notre étude identifie pour la première fois NLRX1 comme un régulateur potentiel de l'homéostasie du glutamate dans le SNC.

Mots-clés: NLRX1; astrocytes; SNC; absorption de glutamate; libération de glutamate; excitotoxicité.

Abstract: Uptake of glutamate from the extracellular space and glutamate release to neurons are two major processes conducted by astrocytes in the central nervous system (CNS) that protect against glutamate excitotoxicity and strengthen neuronal firing, respectively. During inflammatory conditions in the CNS, astrocytes may lose one or both of these functions, resulting in accumulation of the extracellular glutamate, which eventually leads to excitotoxic neuronal death, which in turn worsens the CNS inflammation. NLRX1 is an innate immune NOD-like receptor that inhibits the major inflammatory pathways. It is localized in the mitochondria and was shown to inhibit cell death, enhance ATP production, and dampen oxidative stress. In the current work, using primary murine astrocyte cultures from WT and *Nlrx1*$^{-/-}$ mice, we demonstrate that NLRX1 potentiates astrocytic glutamate uptake by enhancing mitochondrial functions and the functional activity of glutamate transporters. Also, we report that NLRX1 inhibits glutamate release from astrocytes by repressing Ca^{2+}-mediated glutamate exocytosis. Our study, for the first time, identified NLRX1 as a potential regulator of glutamate homeostasis in the CNS.

Keywords: NLRX1; astrocytes; CNS; glutamate uptake; glutamate release; excitotoxicity

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1. Introduction

Astrocytes are the most numerous glial cell type in the central nervous system (CNS). They perform many pivotal functions associated with neuronal support and maintenance of the CNS homeostasis [1]. One of these crucial functions is to uptake excess synaptically-released glutamate (the major excitatory neurotransmitter in the CNS) [2,3] from the extracellular space, metabolize it, and send it back to neurons [4,5]. This glutamate uptake is mediated primarily by the excitatory amino acid transporters 1 and 2 (EAAT1 and EAAT2), expressed by astrocytes (known in mice as glutamate-aspartate transporter (GLAST) and glutamate transporter-1 (GLT-1), respectively) [6–11]. In astrocytes, under physiological conditions, the uptake of glutamate against its concentration gradient relies on glutamate transporters and Na^+/K^+ ATPase that consumes high levels of intracellular adenosine triphosphate (ATP) [12,13]. Many factors in the CNS influence the expression, trafficking, and functional activity of glutamate transporters in astrocytes, including hormones, growth factors, inflammatory mediators, and oxidative stress [14–16].

Recent studies suggested that, along with glutamate uptake, astrocytes release glutamate, which helps synchronize and intensify firing of the surrounding neurons [4,17,18]. Astrocytic glutamate release is mediated mostly by Ca^{2+}-dependent exocytosis [19]. In astrocytes, part of the sequestrated glutamate is transferred into small intracellular vesicles [20,21] by activation of the vesicular glutamate transporters 1 and 2 (VGLUT1 and VGLUT2), derived by the proton gradient generated by the vacuolar (H^+) ATPase (V-ATPase) [22–24]. Astrocytes also express a Ca^{2+} sensor (synaptotagmin 4, 7, or 11) [25–27] and vesicular fusion proteins (vesicle-associated membrane protein 2 and 3 (VAMP2 and VAMP3)) [20,28]. In response to intracellular Ca^{2+} elevation, caused by Ca^{2+} release from the endoplasmic reticulum (ER), vesicular fusion proteins (VAMP2 and VAMP3) fuse with the astrocyte cell membrane fusion proteins (syntaxin and soluble N-ethylmaleimide-sensitive factor attachment protein 23 (SNAP23)) [29–31], resulting in glutamate release from the vesicles into the extracellular space.

In the context of CNS pathologies, astrocytes respond to inflammation by losing some of their vital functions or acquiring some deleterious effects that aggravate inflammatory conditions in the CNS and delay the processes of recovery [1]. Previous studies showed that neuropathological conditions in the CNS, such as brain trauma, infection by human immunodeficiency virus (HIV), multiples sclerosis (MS), amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD), and Parkinson's disease (PD) are associated with accumulation of glutamate in the extracellular space, caused by reduced glutamate uptake and/or increased glutamate release by astrocytes [32–37]. Excess extracellular glutamate induces hyperstimulation of glutamate receptors in neurons and eventually leads to neuronal death, in a process known as "glutamate excitotoxicity" [38]. The excess of extracellular glutamate and subsequent neuronal death, in turn, enhance the inflammatory response and worsen the pathological conditions in the CNS [38].

NOD-like receptors (NLRs) are cytosolic innate immune molecules that can exert either positive or negative effects on inflammation in the CNS [39]. NLRX1 is a recently discovered anti-inflammatory NLR that inhibits nuclear factor-κB (NF-κB) and RIG-1-MAVs signaling pathways, thus inhibiting antimicrobial immune responses [40–44] and sterile inflammations [45]. Since the introduction of *Nlrx1*$^{-/-}$ mice, many studies implicated NLRX1 in the development of various pathologies. For example, *Nlrx1*$^{-/-}$ mice show excessive inflammatory response following Influenza virus infection and LPS treatment [41]. Also, *Nlrx1*$^{-/-}$ mice show exacerbated severity of inflammatory bowel disease (IBD) [46] and increased incidence of colitis-associated colonic

cancer [45]. In the CNS, lack of Nlrx1 in mice is associated with an excessive inflammation following CNS trauma [47], earlier onset, and a more aggressive course of the experimental autoimmune encephalomyelitis (EAE), a mouse model of MS [48]. Moreover, using a neuroblastoma cell line, NLRX1 was shown to inhibit neuronal death and redirect rotenone-treated neurons towards apoptosis instead of necrosis [49]. Unlike other NLRs, NLRX1 is located at the mitochondria. It enhances various mitochondrial functions and activities such as ATP production and respiration while inhibiting oxidative stress and apoptosis [44,49–54].

In the current study, we investigated the role of NLRX1 in glutamate uptake and release by primary murine astrocytes, and the potential mechanisms by which NLRX1 mediates its effects.

2. Materials and Methods

2.1. Mice

All mice handling and manipulations were approved by the Institutional Animal Care and Use Committee at the University of Sherbrooke (Protocols #280-15, 4 April 2017) according to the Canadian Council on Animal Care. All mice were bred on C57/BL6J background. Wild-type (WT) mice were bred in-house in the same conditions as *Nlrx1*$^{-/-}$ mice that were kindly provided by Dr. Jenny P. Y. Ting (Chapel Hill, NC, USA).

2.2. Primary Mouse Astrocyte Cultures

Glial cultures were prepared from 1-day-old pups, as previously described [55]. Pups were sacrificed by decapitation, and brains were harvested and placed in 100 mm culture plates. Brain tissue was dissociated by a commercial razor blade, followed by triturating in 10 mL DMEM/F12 medium (Wisent Inc., Montreal, QC, Canada) containing 10% deactivated fetal bovine serum (dFBS), 2 mM L-glutamine, 1% MEM amino acid, 1% sodium pyruvate, and 1% penicillin-streptomycin and amphotericin B (all from Wisent Inc., Montreal, QC, Canada). Dissociated tissue was passed through 70 µm cell strainer to remove tissue debris. Cells were plated in 100 mm cell culture plates (Corning Inc., Brooklyn, NY, USA) with DMEM/F12 complete medium and incubated in 37 °C incubator with 5% CO_2. The medium was changed every 2–3 days to wash out cells other than glial cells. After 21 days, glial cultures were resuspended in 10% dimethyl sulfoxide (DMSO) in dFBS (freezing medium) and were frozen at −80 °C. One week before the

experiments, cells were thawed and reseeded in 100 mm culture plates, in complete DMEM/F12 medium. Cells were stained with CD11b (eBioscience/Thermofisher scientific, Waltham, Massachusetts, USA # 12-0112-81) as a marker for microglia and the percentage of CD11b-expressing cells was measured by flow cytometry. In our experiments, we used cultures containing less than 10% CD11b+ cells (astrocytes ≥90%) since additional purification of astrocytes did not affect the glutamate uptake or release.

2.3. Glutamate Uptake and Release Assay

The assay was modified from Piao et al. 2015 [56]. 100,000 astrocytes were seeded in each well of a 96-well plate, and washed 2 times with Hank's Balanced Salt Solution (HBSS) containing Ca^{2+} (Wisent Inc., Montreal, QC, Canada): 1.26 mM $CaCl_2$ (anhydrous), 5.36 mM KCl, 0.44 mM KH_2PO_4, 0.811 mM $MgSO_4$ (anhydrous), 137 mM NaCl, 0.336 mM Na_2HPO_4 (anhydrous), 4.166 mM $NaHCO_3$, and 5.55 mM D-glucose, pH 7.25 ± 0.15 or Ca^{2+}-free Lock's solution: 140 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 $MgSO_4$, 11 mM glucose, and 15 mM HEPES-NaOH. For glutamate uptake, cells were incubated with 100 or 200 µM glutamate in the Ca^{2+}-containing HBSS for 4 h, while for glutamate release, astrocytes were incubated in Ca^{2+}-containing HBSS or Ca^{2+}-free Lock's solution for 1 h, in the 37 °C with 5% CO_2 incubator. Then, culture supernatant was collected, and glutamate concentration in the medium was measured using a glutamate colorimetric assay kit (Sigma-Aldrich, Oakville, ON, Canada # MAK004 or Abcam, Toronto, ON, Canada #ab83389) according to the manufacturer's instructions. Glutamate uptake by astrocytes was measured by subtracting the amount of glutamate measured in the medium from the amount initially added to the cells. Both glutamate uptake and release by astrocytes were normalized to the amount of protein in the corresponding cells, measured by Bradford assay (Bio-Rad, Montreal, QC, Canada).

2.4. Quantitative Real-Time PCR (qPCR)

RNA was extracted from astrocytes using TRIzol reagent (Life Technologies Inc./Thermofisher Scientific, Waltham, Massachusetts, USA # 15596-018) according to the manufacturer's instructions. cDNA was synthesized from RNA, using Oligo(dT) primer (IDT, Coralville, Iowa, USA), dNTP Mix, M-MuLV Reverse Transcriptase, M-MuLV Reverse Transcriptase Buffer, and RNase inhibitor (all from New England Biolabs, Whitby, ON, Canada),

as previously described [55]. qPCR was performed using KiCqStart® SYBR® Green qPCR ReadyMix (Sigma-Aldrich, Oakville, ON, Canada # KCQS00). The $\Delta\Delta C_T$ method was used to calculate the relative gene expression to *18S* as a housekeeping gene [57]. Primer sequences used (IDT, Coralville, Iowa, USA) are shown in Table 1.

Table 1. Primer sequences used for qPCR.

18S	F: 5′ CGG CTA CCA CAT CCA AGG AA ′3 R: 5′ GCT GGA ATT ACC GCG GCT ′3
Exocytosis	
V-ATPase d2	F: 5′ TTC AGT TGC TAT CCA GGA CTC GGA ′3 R: 5′ GCA TGT CAT GTA GGT GAG AAA TGT GCT CA ′3
VGLUT1	F: 5′ GGT GGA GGG GGT CAC ATA C ′3 R: 5′ AGA TCC CGA AGC TGC CAT AGA ′3
VGLUT2	F: 5′ CCC TGG AGG TGC CTG AGA A ′3 R: 5′ GCG GTG GAT AGT GCT GTT GTT ′3
Synaptotagmin 11	F: 5′ GAC ACT TGC CGA AGA TGG ATA TC ′3 R: 5′ TGC GTT TTC TGC CGT AGT AGA ′3
VAMP2	F: 5′ CAC AAT CTG GTT CTT TGA GGA G ′3 R: 5′ AGA GAC TTC AGG CAG GAA TTA G ′3
VAMP3	F: 5′ CTC ACC AAG GCA TCA GTC TG ′3 R: 5′ ATT CTA AGA GCA CCA GGC ATC ′3
Syntaxin 1a	F: 5′ TCC AAG CTA AAG AGC ATT GAG C ′3 R: 5′ GGC GTT GTA CTC GGA CAT GA ′3
SNAP23	F: 5′ AAT CCT GGG TTT AGC CAT TGA GTC ′3 R: 5′ TTG GTC CAT GCC TTC TTC TAT GC ′3
Glutamate transporters	
GLT-1	F: 5′ CGA TGA GCC AAA GCA CCG AA ′3 R: 5′ CTG GAG ATG ATA AGA GGG AGG ATG ′3
GLAST	F: 5′ TCA AGT TCT GCC ACC CTA CC ′3 R: 5′ TCT GTC CAA AGT TCA GGT CAA ′3

2.5. Flow Cytometry Staining

To measure the total protein expression of glutamate transporters, intracellular staining was performed (protocol modified from Gharagozloo et al. 2018 and Schwarz et al. 2013) [58,59]. WT and *Nlrx1*$^{-/-}$ astrocytes were washed with phosphate-buffered saline (PBS), fixed, permeabilized, and blocked with 5% dFBS in washing buffer. Cells were stained with the anti-GLT-1 antibody (Novus Biologicals, Centennial, CO, USA # NBP1-20136) diluted 1:100 or anti-GLAST antibody (Novus Biologicals, Centennial, CO, USA # NB100-1869) diluted 1:200 and incubated for 30–40 min. Astrocytes were washed twice and incubated with the secondary anti-rabbit IgG antibody, Alexa Fluor® 555 Conjugated (New England Biolabs, Whitby, ON, Canada #4413) diluted 1:1000, for 20 min. Cells were washed twice after the secondary antibody and resuspended in PBS.

To measure the cell surface expression of the transporters, astrocytes were washed and stained using the same previous procedure, but with no cell fixation or permeabilization.

To detect the activity of reactive oxygen species (ROS), dihydrorhodamine 123 (DHR) was added to the cells to a final concentration of 0.5 µg/mL and incubated for 15 min at 37 °C, then cells were resuspended in PBS (protocol modified from Gris et al. 2008 and Farrell et al. 2011) [60,61].

Sample acquisition was realized using Beckman Coulter CytoFlex (Beckman Coulter, Brea, CA, USA). Data analysis was performed, and histograms produced using CytExpert 2.3 software (Beckman Coulter, Brea, CA, USA).

2.6. Measurement of Intracellular ATP

The assay was performed using the ATP bioluminescent assay kit (Sigma-Aldrich, Oakville, ON, Canada # FLAA) according to the manufacturer's instructions, modified from Marcaida et al. 1997 [62]. 100,000 astrocytes from WT and *Nlrx1*$^{-/-}$ mice were lysed with 400 µl of somatic cell ATP-releasing reagent (Sigma-Aldrich, Oakville, ON, Canada # FLSAR). In a white opaque 96-well plate, 100 µl of the ATP reaction mix was added to each well and incubated for 3 min. Samples (100 µl) from the ATP standard or the cell lysate were added to the reaction mix and vigorously mixed. Immediately, using a luminometer, the amount of light produced from the reaction was measured, which reflected the amount of ATP in each well.

2.7. Measurement of Mitochondrial DNA (mtDNA)

DNA was extracted from astrocytes using TRIzol reagent (Life Technologies Inc./Thermofisher Scientific, Waltham, Massachusetts, USA # 15596-018) according to the manufacturer's instructions. qPCR was performed, as previously described, to compare the relative amount of mtDNA between WT and *Nlrx1*$^{-/-}$ astrocytes [63], using 100 ng of the extracted DNA and KiCqStart® SYBR® Green qPCR ReadyMix (Sigma-Aldrich, Oakville, ON, Canada # KCQS00). To estimate the amount of mtDNA, the mitochondrial DNA region (*D-loop*) was amplified, using the two primer sequences: D1 (5′-CCC AAG CAT ATA AGC TAG TAC-3′) and D2 (5′-ATA TAA GTC ATA TTT TGG GAA CTA C-3′), with the thermal cycling protocol 95 °C for 20 s, 55 °C for 20 s, 72 °C for 80 s for 30 cycles after an initial denaturation. To estimate the amount of nuclear DNA as a reference, the (*apo-B*) region was amplified, using the two primer sequences: 5′-CGT GGG CTC CAG CAT TCT A-3′ and 5′-TCA CCA GTC ATT TCT GCC TTT G-3′, with the two-step thermal cycling protocol 95 °C for 10 s and 60 °C for 30 s for 40 cycles after an initial denaturation at 95 °C for 1 min. The relative amount of mtDNA to the nuclear DNA was calculated by the $\Delta\Delta C_T$ method.

2.8. Statistical Analysis

All statistical analysis was conducted using GraphPad Prism 8 software (GraphPad, San Diego, CA, USA). Results were expressed as the mean ± standard error of the mean (SEM). Statistical differences between WT and *Nlrx1*$^{-/-}$ astrocytes were assessed by Mann–Whitney test. Glutamate uptake and glutamate release using different treatments were assessed by two-way ANOVA followed by Tukey's test. The significance level was set at $p < 0.05$.

3. Results

3.1. NLRX1 Inhibits Excess Glutamate Release and Enhances Glutamate Uptake by Astrocytes

To determine the role of NLRX1 in the glutamate release and uptake by astrocytes, we incubated primary astrocyte cultures from WT and *Nlrx1*$^{-/-}$ mice with or without glutamate in a Ca^{2+}-containing medium. Our data shows that in the cultures incubated with the glutamate-free medium for 4 h (Figure 1A) or 1 h (Figure 2A), *Nlrx1*$^{-/-}$ astrocytes released significantly higher levels of glutamate compared to WT astrocytes. In the cultures incubated with 100 µM glutamate,

there was no significant difference in the glutamate uptake between WT and *Nlrx1*$^{-/-}$ astrocytes (Figure 1B). However, when we challenged both cultures with a higher concentration of glutamate (200 µM), WT astrocytes significantly enhanced their glutamate uptake, while there was no significant change in the *Nlrx1*$^{-/-}$ astrocytes' glutamate uptake (Figure 1B). Therefore, after incubation with 200 µM glutamate, WT astrocytes had a significantly higher glutamate uptake (fivefold) than *Nlrx1*$^{-/-}$ astrocytes (Figure 1B).

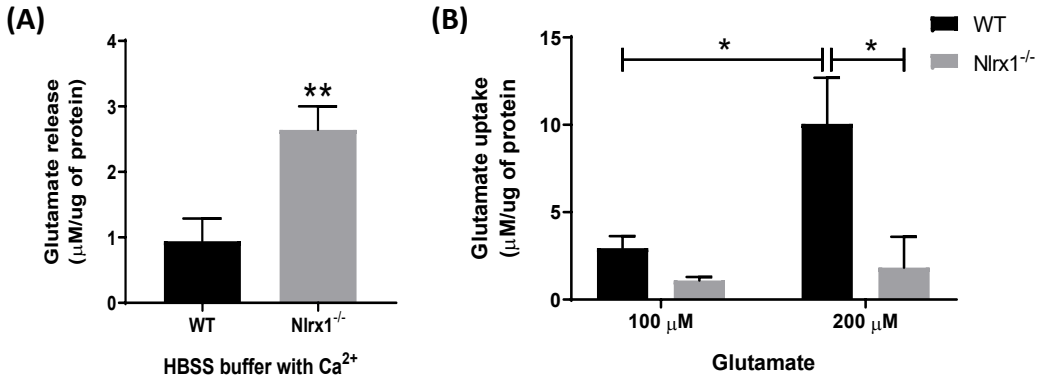


Figure 1. NLRX1 inhibits glutamate release and enhances glutamate uptake by astrocytes. (**A**) WT and *Nlrx1*$^{-/-}$ astrocyte cultures were incubated in glutamate-free, Ca^{2+}-containing HBSS medium. After 4 hrs, the culture supernatant was collected, and glutamate release in the medium was measured ($n = 7$), ** $p < 0.01$ as determined by Mann–Whitney test. (**B**) For evaluation of glutamate uptake, astrocyte cultures were incubated with 100 or 200 µM glutamate in the Ca^{2+}-containing HBSS medium ($n = 5$), * $p < 0.05$ as determined by Tukey's test, results are presented as mean ± SEM.

3.2. Nlrx1$^{-/-}$ *Astrocytes' Excess Glutamate Release Is* Ca^{2+}*-Dependent*

Given that glutamate release by astrocytes is mediated primarily by the elevation of intracellular Ca^{2+} levels [18], first, we examined whether the presence of extracellular Ca^{2+} plays a role in glutamate release from *Nlrx1*$^{-/-}$ astrocytes. We incubated WT and *Nlrx1*$^{-/-}$ astrocyte cultures in a Ca^{2+}-containing or Ca^{2+}-free medium for 1 h, followed by the measurement of glutamate in the medium. We observed that removal of Ca^{2+} from the medium resulted in a significant increase in glutamate release from WT astrocytes, while no significant changes in glutamate release were observed in the *Nlrx1*$^{-/-}$ astrocytes (Figure 2A). This suggests that the presence of extracellular Ca^{2+} does not have a significant effect on glutamate release in *Nlrx1*$^{-/-}$ cultures.

We further assessed whether this glutamate release is mediated by Ca^{2+} release from the intracellular Ca^{2+} stores, including ER and mitochondria. We incubated WT and *Nlrx1*$^{-/-}$ astrocyte cultures with different concentrations of 2-Aminoethyl diphenylborinate (2-APB, an inhibitor of inositol-1,4,5-trisphosphate (IP3) receptors that inhibits Ca^{2+} release from the ER) [64] or Cyclosporin A (CsA, an inhibitor of mitochondrial Ca^{2+} release) [65] in the Ca^{2+}-free medium. A significant reduction was detected in the glutamate release from 2-APB-treated *Nlrx1*$^{-/-}$ astrocytes at both concentrations, compared to WT (Figure 2B). By contrast, in cultures treated with CsA, no significant change was detected in both genotypes (Figure 2C).

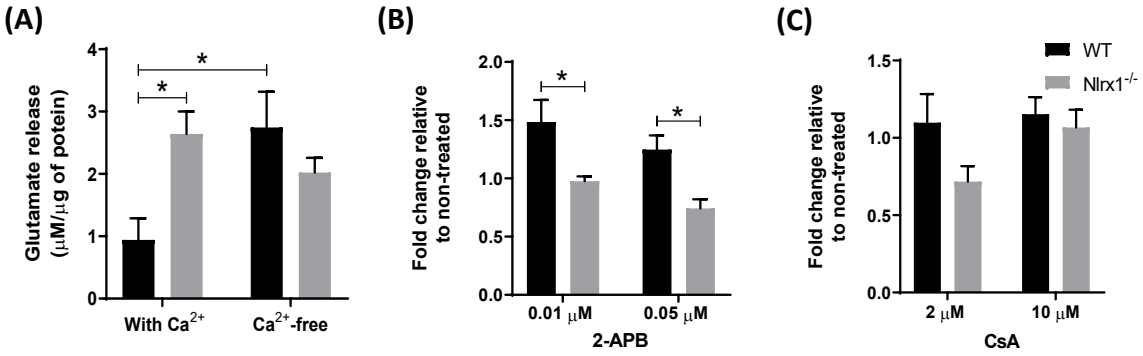


Figure 2. Excess Ca^{2+} release from the ER mediates glutamate release from *Nlrx1*$^{-/-}$ astrocytes. (**A**) WT and *Nlrx1*$^{-/-}$ astrocytes were incubated in a Ca^{2+}-containing HBSS buffer or Ca^{2+}-free Lock's solution for 1 h (n = 7); (**B**) astrocyte cultures were incubated with 0.01 or 0.05 μM of 2-APB (n = 5), or (**C**) with 2 or 10 μM of CsA (n = 6) in the Ca^{2+}-free Lock's solution for 1 h. The supernatant was collected from all cultures and glutamate in the medium was measured by the glutamate assay kit. * $p < 0.05$ as determined by Tukey's test, results are presented as mean ± SEM.

3.3. Glutamate Release by Nlrx1$^{-/-}$ *Astrocytes Is Mediated By Exocytosis*

Since we found that the NLRX1-mediated glutamate release is Ca^{2+}-dependent, we further evaluated whether it is mediated by exocytosis. We measured gene expression of the proteins involved in exocytosis, upstream and downstream of the Ca^{2+} release from the ER, in WT and *Nlrx1*$^{-/-}$ astrocyte cultures. The results demonstrated that the mRNA expression of the astrocytic Ca^{2+} sensor, synaptotagmin 11, and the vesicular fusion proteins (VAMP2 and VAMP3) was significantly upregulated in *Nlrx1*$^{-/-}$ astrocytes relative to WT (Figure 3A), while no significant change was observed in the mRNA expression of the cell membrane fusion proteins (Syntaxin 1a

and SNAP23) (Figure 3B) or the proteins upstream of the Ca^{2+} release (V-ATPase d2, VGLUT1, and VGLUT2) (Figure 3C).

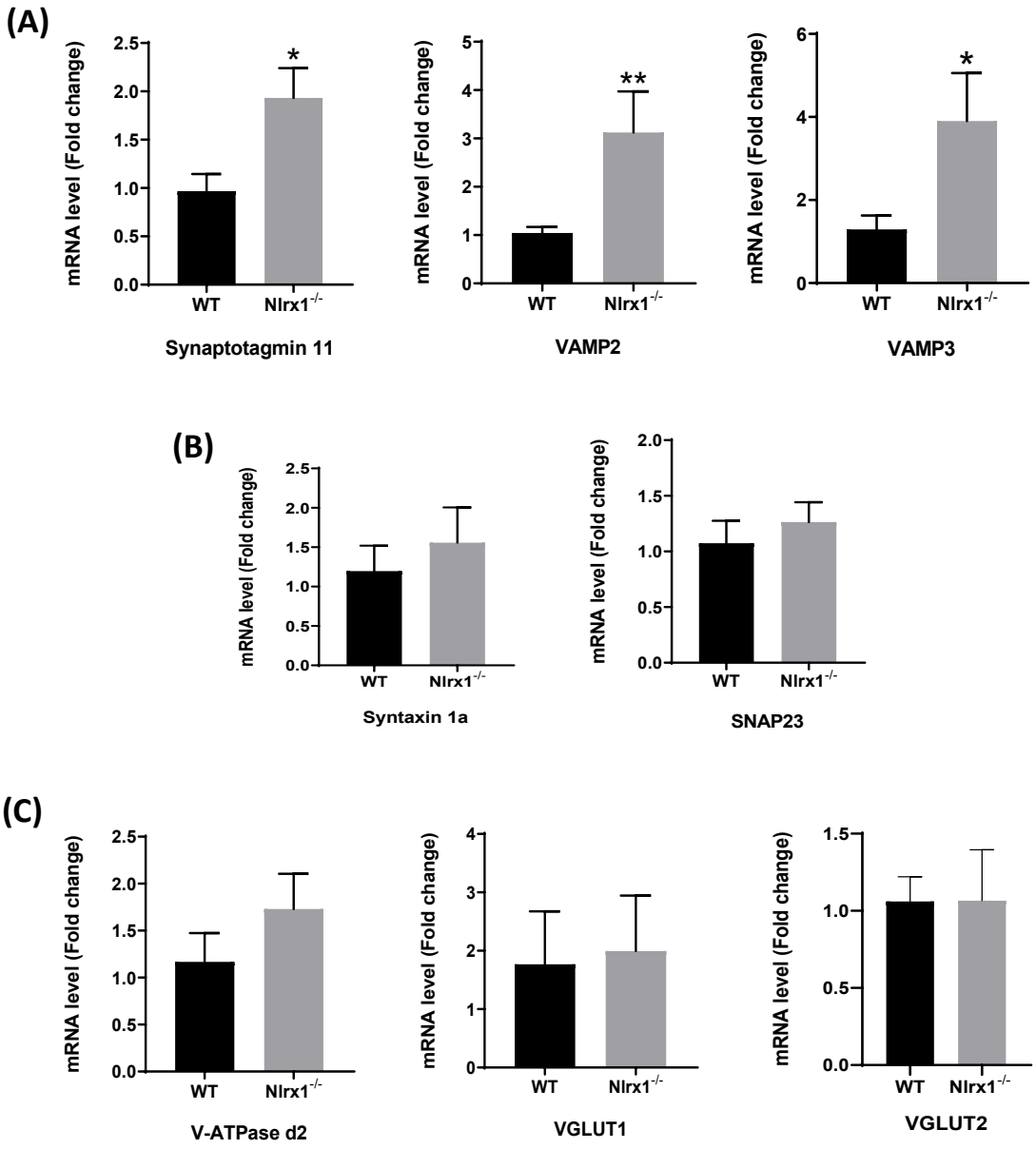
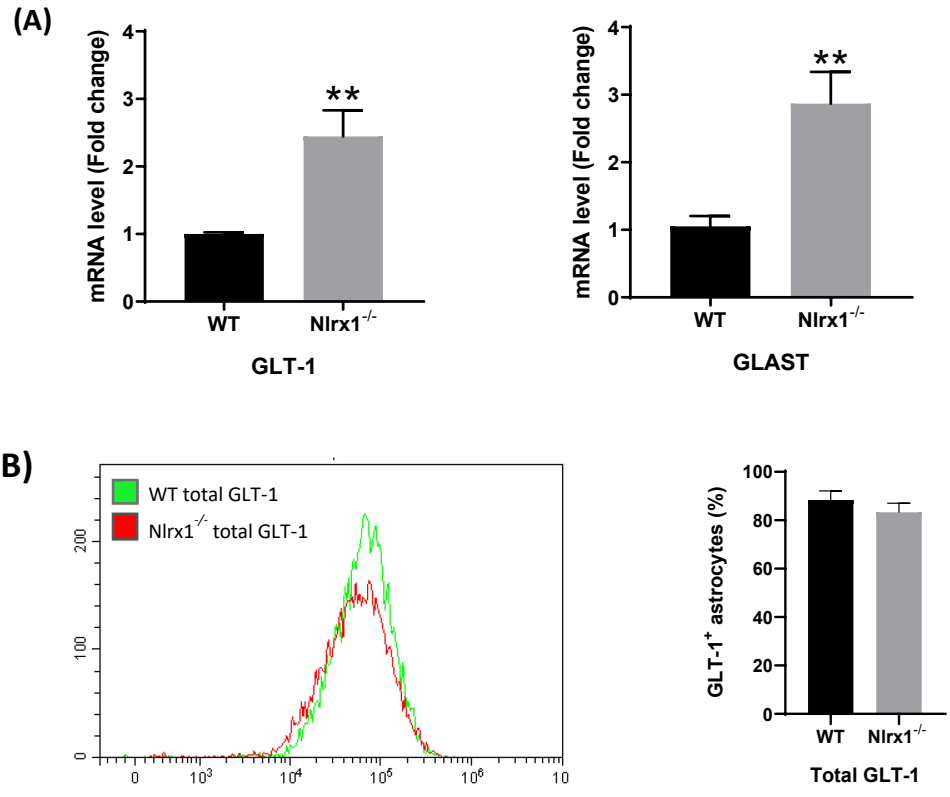


Figure 3. Increased mRNA expression of the proteins of exocytosis in *Nlrx1*$^{-/-}$ astrocytes. mRNA expression of the proteins of exocytosis in WT and *Nlrx1*$^{-/-}$ astrocytes shows (**A**) significant upregulation of mRNA expression of the Ca^{2+} sensor, synaptotagmin 11, and vesicular fusion proteins, VAMP2 and VAMP3, in *Nlrx1*$^{-/-}$ astrocytes compared to WT; (**B**) no significant change in the cell membrane fusion proteins' mRNA expression; and (**C**) no significant change in the expression of the proteins upstream of Ca^{2+} release from the ER. ** $p < 0.01$ and * $p < 0.05$ as determined by Mann–Whitney test ($n \geq 5$), results are presented as mean ± SEM.

3.4. mRNA and Protein Expression of Glutamate Transporters in Astrocytes

To further investigate the mechanism by which NLRX1 enhances glutamate uptake, we measured the relative gene expression of the astrocytes' glutamate transporters, GLT-1 and GLAST, in WT and *Nlrx1*$^{-/-}$ astrocyte cultures, using qPCR. The mRNA expression of both transporters was significantly higher in *Nlrx1*$^{-/-}$ astrocyte cultures relative to WT (Figure 4A). In parallel, we stained WT and *Nlrx1*$^{-/-}$ astrocytes with anti-GLT-1 or anti-GLAST antibodies and quantified the total protein expression and the cell surface expression of both transporters by flow cytometry. As shown in Figure 4B and 4C, no significant change was detected in either GLT-1 or GLAST total protein expression (Figure 4B) or cell surface expression (Figure 4C) between astrocytes of both genotypes.



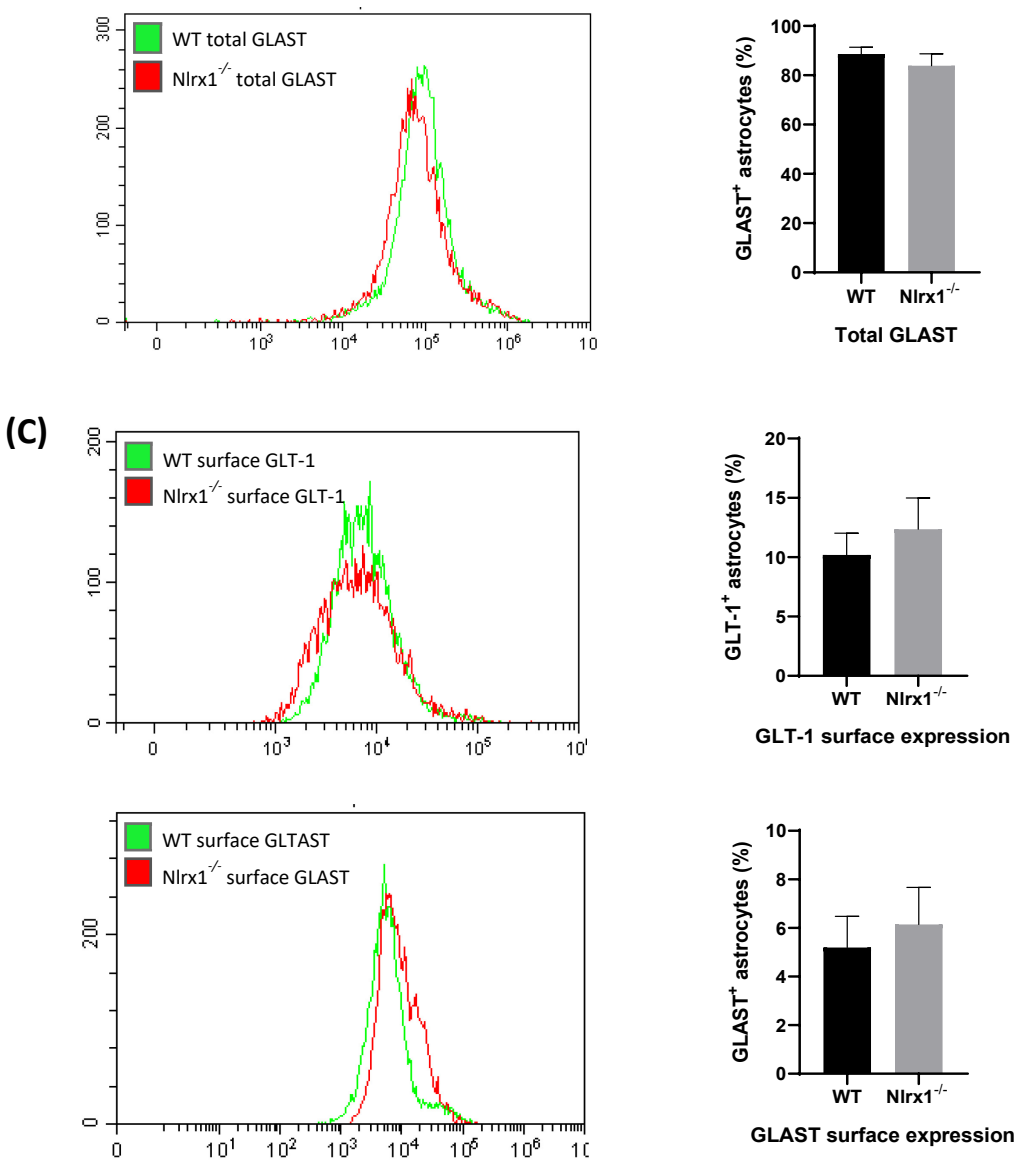


Figure 4. mRNA and protein expression of GLT-1 and GLAST in astrocytes. (**A**) mRNA expression of GLT-1 and GLAST is significantly upregulated in *Nlrx1*$^{-/-}$ astrocytes compared to WT (n = 5). ** $p < 0.01$ as determined by Mann–Whitney test; (**B**) the total protein expression of GLT-1 and GLAST proteins in WT and *Nlrx1*$^{-/-}$ astrocytes was measured by flow cytometry (n = 5); (**C**) the cell surface expression of both transporters on astrocytes was measured by flow cytometry (n = 7). Representative flow cytometric histograms presented on the left side, $p > 0.05$ as determined by Mann–Whitney test, results are presented as mean ± SEM.

3.5. NLRX1 Enhances Mitochondrial Functions in Astrocytes

The optimal function of glutamate transporters requires an enormous amount of energy [12,13]. Accordingly, we measured levels of intracellular ATP in both WT and *Nlrx1*$^{-/-}$ astrocytes. We found that *Nlrx1*$^{-/-}$ astrocytes have significantly less (20%) intracellular ATP compared to WT

(Figure 5A). Since oxidative stress exerts a negative effect on the functional activity of the transporters [66,67], we measured ROS activity in both WT and *Nlrx1*$^{-/-}$ astrocytes by flow cytometry. Our results demonstrated that *Nlrx1*$^{-/-}$ astrocytes have significantly higher oxidative activity than WT (Figure 5B). Since mitochondria are the major source of intracellular ATP and ROS in the cells, we evaluated the number of mitochondria in WT and *Nlrx1*$^{-/-}$ astrocytes. No significant difference was detected in the amount of mtDNA between WT and *Nlrx1*$^{-/-}$ astrocytes (Figure 5C).

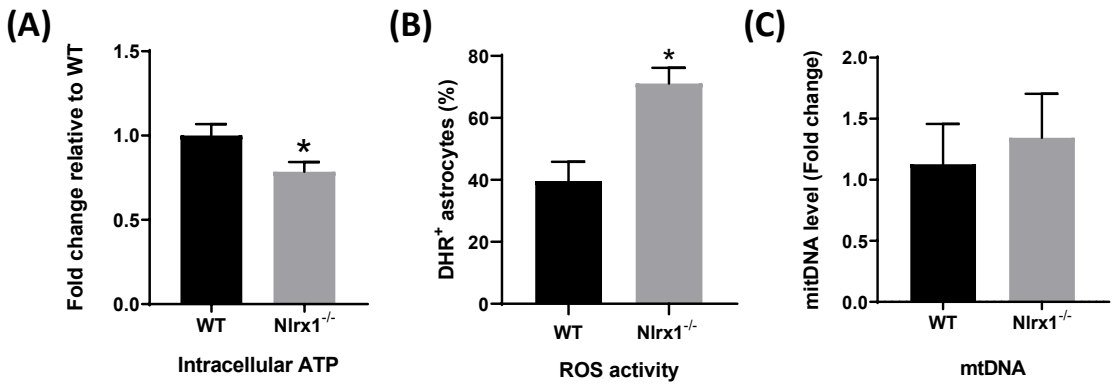


Figure 5. NLRX1 enhances mitochondrial functions in astrocytes. (**A**) The level of intracellular ATP was measured in WT and *Nlrx1*$^{-/-}$ astrocytes using an ATP bioluminescent assay kit (n = 5); (**B**) the level of oxidative activity was measured in WT and *Nlrx1*$^{-/-}$ astrocytes by flow cytometry (n = 4); (**C**) the difference between the amount of mtDNA in WT and *Nlrx1*$^{-/-}$ astrocytes was measured by qPCR (n = 3). * $p < 0.05$ as determined by Mann–Whitney test, results are presented as mean ± SEM.

4. Discussion

In this study, we report that the anti-inflammatory [40–45,47,48] and prosurvival molecule [49], NLRX1, helps maintain glutamate homeostasis in the CNS. Our findings suggest that NLRX1 enhances astroglial glutamate uptake by promoting the functional activity of glutamate transporters and inhibits glutamate release from astrocytes by suppressing Ca^{2+}-mediated glutamate exocytosis. To our knowledge, this is the first time that one protein has been shown to be implicated in both processes that regulate glutamate homeostasis.

Given that GLT-1 and GLAST are responsible for the uptake of more than 90% of the extracellular glutamate in the CNS [68,69], we measured the mRNA and protein expression of these glutamate transporters in astrocytes from WT and *Nlrx1*$^{-/-}$ mice. Unexpectedly, the mRNA expression of both transporters was significantly higher in *Nlrx1*$^{-/-}$ astrocytes than WT. However,

when we measured the total protein expression, as well as the cell surface expression of both transporters, we did not see any significant difference between astrocytes of both genotypes. These findings suggest that the enhanced glutamate uptake in WT astrocytes cannot be attributed to changes in the transcription or translation of the glutamate transporters. In line with our findings, Conrad and Stoffel reported that the direct phosphorylation of GLAST protein by protein kinase C (PKC) reduces its glutamate uptake activity, while immunofluorescence does not show any effect on its protein expression [70]. Another study revealed that arachidonic acid (AA) downregulates glutamate uptake by EAAT-1 by decreasing its affinity to glutamate and the maximal transport rate approximately 30% with no effect on the expression of its protein [71]. In a third study, Trotti et al. reported that oxidative stress by H_2O_2 induces direct oxidation of the sulfhydryl (SH) group of both transporters, which decreases their glutamate uptake with no protein degradation or reduction in their surface expression [72].

Since NLRX1 is localized in the mitochondria, we hypothesized that NLRX1 enhances the glutamate uptake activity of both GLT-1 and GLAST by improving mitochondrial functions in astrocytes. The level of intracellular ATP in astrocytes is one of the crucial factors that determine the functional activity of glutamate transporters [12,13]. Many previous studies reported that ATP depletion, as in cases of brain ischemia, induces glutamate uptake failure caused by loss or reversal of the transporters' function [73,74]. To pay for its own energy consumption, a large portion of glutamate metabolites in astrocytes is consumed in the tricarboxylic acid (TCA) cycle to produce more ATP [75]. Interestingly, we found that *Nlrx1*$^{-/-}$ astrocytes contain 20% less ATP that WT astrocytes, which could either be a cause and/or a result of the reduced glutamate uptake by these cells. Nevertheless, these data, in agreement with the previous studies [50,51], suggest that NLRX1 enhances mitochondrial ATP production.

Moreover, it was reported that the intracellular oxidative stress induced by H_2O_2 reduces the functional activity of glutamate transporters [66,67]. In this regard, we measured the level of ROS activity in both WT and *Nlrx1*$^{-/-}$ astrocytes. Our results demonstrated that *Nlrx1*$^{-/-}$ astrocytes have 50% more oxidative activity than WT, which could be responsible for the significant deficiency of their glutamate uptake. Furthermore, since there was no difference in the level of mtDNA between WT and *Nlrx1*$^{-/-}$ astrocytes, we excluded the possibility that NLRX1 increases the number of mitochondria.

Taken together, our results suggest that NLRX1 enhances mitochondrial functions in astrocytes, and thus boosts the functional activity of both GLT-1 and GLAST, rather than their protein expression. This effect of NLRX1 on the transporters is achieved by suppressing oxidative stress and, partially, by maintaining sufficient ATP production.

Recent studies suggest that astrocytes express components necessary for the Ca^{2+}-mediated exocytosis, which is the principal mechanism of astroglial glutamate release under physiological conditions [4,17–19]. We evaluated whether the excess glutamate release from *Nlrx1*$^{-/-}$ astrocytes is Ca^{2+}-dependent. First, to exclude the role of extracellular Ca^{2+}, we incubated astrocytes in a Ca^{2+}-free instead of the Ca^{2+}-containing medium. We observed that WT astrocytes significantly upregulated their glutamate release after Ca^{2+} removal, which agrees with the previously published report by Kostic et al. (2017) [76]. The removal of Ca^{2+} from the medium stimulates Ca^{2+} release from the intracellular Ca^{2+} stores and results in augmentation of glutamate release from astrocytes [76]. However, this does not explain the phenotype in *Nlrx1*$^{-/-}$ astrocytes, as there was no change between their glutamate release in the Ca^{2+}-containing and the Ca^{2+}-free media, probably because they had already reached their maximum capacity of glutamate release, and Ca^{2+} removal does not result in any additional effect. In the second step, we evaluated whether this glutamate release occurs in response to Ca^{2+} release from the intracellular Ca^{2+} stores. We found that inhibiting Ca^{2+} release from the mitochondria does not exhibit any significant effect while inhibiting Ca^{2+} release from the ER reduces the excess glutamate release from *Nlrx1*$^{-/-}$ astrocytes. These observations corroborate previous findings that ER Ca^{2+} plays an essential role in glutamate release [4,17].

Consequently, *Nlrx1*$^{-/-}$ astrocytes show higher mRNA expression of exocytosis proteins downstream of Ca^{2+} release from the ER, including the Ca^{2+} sensor, synaptotagmin 11, and the vesicular fusion proteins (VAMP2 and VAMP3). In contrast, there is no significant change in the expression of the cell membrane fusion proteins (Syntaxin 1 a and SNAP23), or the proteins preceding Ca^{2+} release from the ER (V-ATPase d2, VGLUT1, and VGLUT2). Therefore, our results suggest that the excess glutamate release from *Nlrx1*$^{-/-}$ astrocytes is mediated by excess Ca^{2+} release from the ER, followed by an augmentation in the expression of the molecules of exocytosis as a result of the excess Ca^{2+} release. Collectively, these data provide evidence that NLRX1 mediates its inhibitory effect on glutamate release from astrocytes mainly by suppressing Ca^{2+} release from the ER, which consequently suppresses glutamate exocytosis.

The connection between the two mechanisms by which NLRX1 mediates its effects on astroglial glutamate uptake and release is still unclear. Being situated in the mitochondria, it is plausible that NLRX1 modifies mitochondrial functions [44,49–51]. However, the mechanism by which NLRX1 modifies the function of the ER requires more in-depth investigations.

In conclusion, in the current study, we provide evidence that NLRX1 enhances astroglial glutamate uptake and inhibits excess glutamate release from astrocytes, thus maintaining glutamate homeostasis in the CNS (Figure 6 - modified from the graphical abstract of our recent publication) [4]. Consequently, NLRX1 represents a potential therapeutic target for the inflammatory and neurodegenerative diseases associated with glutamate excitotoxicity in the CNS.

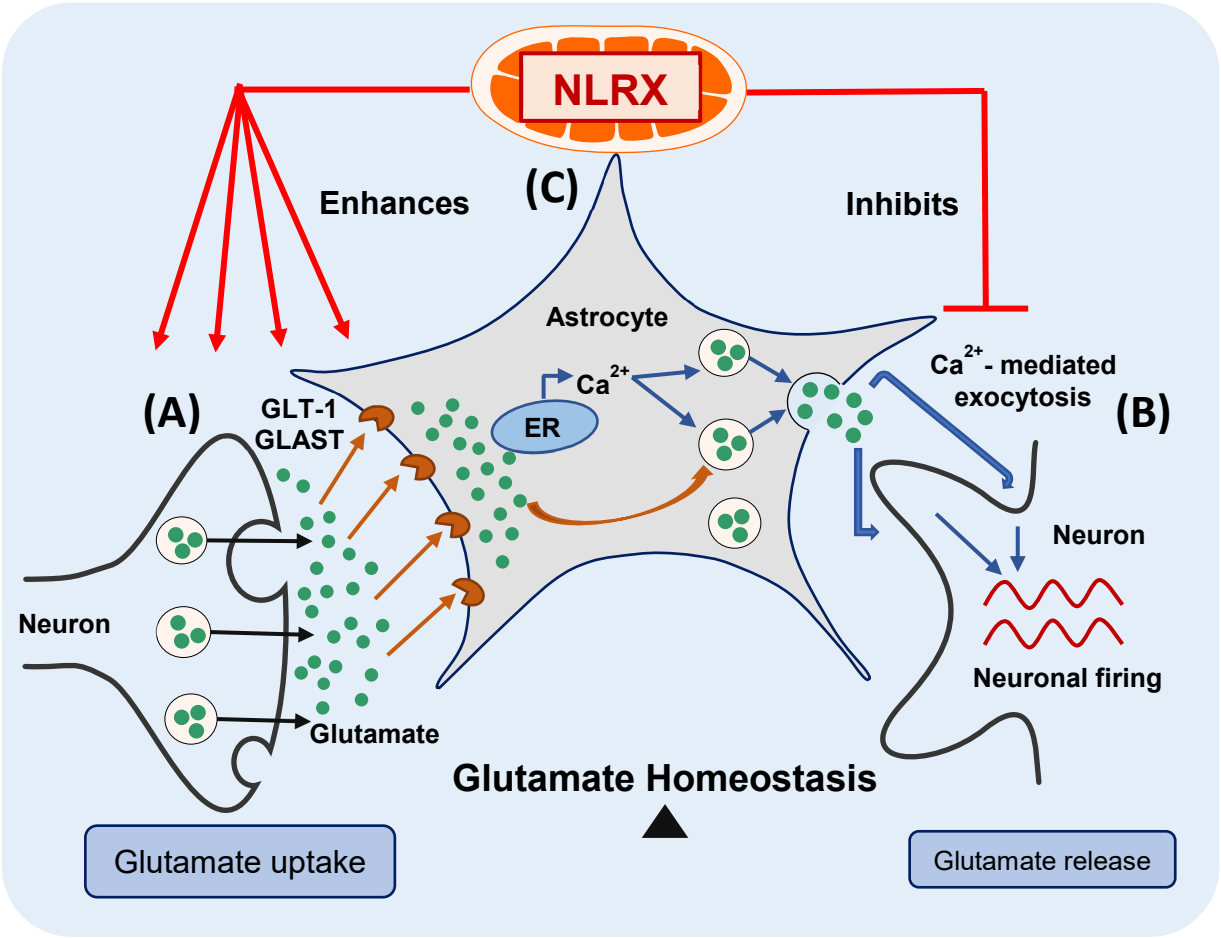


Figure 6. NLRX1 maintains glutamate homeostasis in the CNS. (**A**) Uptake of the extracellular glutamate by astrocytes is mediated by the glutamate uptake transporters (GLT-1 and GLAST); (**B**) Ca^{2+}-mediated exocytosis mediates glutamate release from astrocytes, in response to Ca^{2+} release from the ER. The released glutamate helps to synchronize and reinforce the firing of the surrounding neurons; (**C**) NLRX1 enhances astroglial glutamate uptake and

inhibits its Ca^{2+}-mediated glutamate exocytosis, hence maintaining glutamate homeostasis in the CNS.

Author contributions: S.M., A.A., and D.G. designed the study and the experiments. S.M. performed the experiments and the statistical analysis and wrote the manuscript. M.G. and C.S. as second coauthors helped with animal care, genotyping, and culture preparation. M.G. participated in the flow cytometry experiments and their analysis. C.S. performed the ROS experiment by flow cytometry and its analysis. C.S., A.A., and D.G. contributed to the conceptual reading and critical editing of the manuscript. All authors read and approved the manuscript.

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Abbreviations

CNS	Central nervous system
EAAT1 and EAAT2	Excitatory amino acid transporters 1 and 2
GLAST	Glutamate–aspartate transporter
GLT-1	Glutamate transporter-1
ATP	Adenosine triphosphate
VGLUT1 and VGLUT2	Vesicular glutamate transporters 1 and 2
V-ATPase	Vacuolar (H+) ATPase
VAMP2 and VAMP3	Vesicle-associated membrane protein 2 and 3
ER	Endoplasmic reticulum
SNAP23	Soluble N-ethylmaleimide-sensitive factor attachment protein 23
HIV	Human immunodeficiency virus
MS	Multiple sclerosis
ALS	Amyotrophic lateral sclerosis
AD	Alzheimer's disease
PD	Parkinson's disease
NLRs	NOD-like receptors
NF-κB	Nuclear factor-κB
NLRX1	NOD-like receptor X1

IBD	Inflammatory bowel disease
EAE	Experimental autoimmune encephalomyelitis
WT	Wild-type
Nlrx1$^{-/-}$	Nlrx1 knockout
dFBS	Deactivated fetal bovine serum
DMSO	Dimethyl sulfoxide
HBSS	Hank's Balanced Salt Solution
qPCR	Quantitative real-time PCR
PBS	Phosphate-buffered saline
ROS	Reactive oxygen species
DHR	Dihydrorhodamine 123
mtDNA	Mitochondrial DNA
SEM	Standard error of the mean
2-APB	2-Aminoethyl diphenylborinate
IP3	Inositol-1,4,5-trisphosphate
CsA	Cyclosporin A
PKC	Protein kinase C
AA	Arachidonic acid
SH group	Sulfhydryl group
TCA	Tricarboxylic acid

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4. DISCUSSION

Inflammatory conditions in the CNS are often complicated by glutamate excitotoxicity. During inflammation, reactive astrocytes may lose their function of glutamate uptake and/or release an excess amount of glutamate to the extracellular space. The resulting accumulating glutamate in the extracellular space is toxic to neurons, that eventually induces neuronal death. Neuronal death as a result of glutamate excitotoxicity reactivates the resident CNS cells and attracts more inflammatory cells to the CNS, which worsens inflammation and delays the recovery process (Dong et al. 2009; Wang and Qin 2010). Our molecule of interest, NLRX1, is an anti-inflammatory molecule in the CNS (Eitas et al. 2014; Theus et al. 2017) and a neuro-survival factor (Imbeault et al. 2014). Based on that, we investigated the role of NLRX1 in astroglial glutamate uptake and release, using primary murine astrocyte cultures from WT and *Nlrx1*$^{-/-}$ mice. Our results demonstrate, for the first time, that one protein, NLRX1, supports glutamate homeostasis mediated by astrocytes. We showed that NLRX1 enhances astroglial glutamate uptake and inhibits glutamate release from astrocytes. Therefore, we identify NLRX1 as a potential inhibitor of glutamate excitotoxicity in the CNS.

4.1. Effect of NLRX1 on glutamate release by astrocytes

Astroglial glutamate release is a recently discovered function of astrocytes in the CNS, which represents a part of the gliotransmitter release. The released glutamate from astrocytes modulates neurotransmitter release from the adjacent inhibitory or excitatory neurons and strengthens their firings. Yet, very little is known about the regulatory factors that stimulate or inhibit this astroglial function. By contrast, many studies identified various mechanisms mediating astroglial glutamate release. Among which, Ca^{2+}-mediated exocytosis is the main proposed mechanism by which astrocytes release glutamate (Hamilton and Attwell 2010; Mahmoud et al. 2019; Malarkey and Parpura 2008; Parpura and Haydon 2000). In our experiments, when we incubated WT and *Nlrx1*$^{-/-}$ astrocytes in a Ca^{2+}-containing medium, *Nlrx1*$^{-/-}$ astrocytes released more than double the amount of glutamate released by WT astrocytes. As a first step, we assessed whether this excess glutamate release from *Nlrx1*$^{-/-}$ astrocytes is Ca^{2+}-dependent.

4.1.1. Extracellular Ca^{2+} does not play a role in NLRX1-mediated astroglial glutamate release

In work done by Hua and coworkers, using primary rat cortical astrocytes, they revealed that entrance of the extracellular Ca^{2+} to astrocytes may play a minor role in increasing the level of the $[Ca^{2+}]_i$ and the subsequent glutamate release by astrocyte cultures. When they blocked Ca^{2+} entry by Cd^{2+}, this resulted in a reduction of glutamate release from the astrocyte cultures (Hua et al. 2004). Accordingly, to verify if extracellular Ca^{2+} plays a role in glutamate release in our case, we incubated WT and *Nlrx1*$^{-/-}$ astrocytes in a Ca^{2+}-free medium. Unlike the results obtained by Hua et al., after Ca^{2+} removal, WT astrocytes upregulated their glutamate release to almost triple the amount of glutamate they released in the Ca^{2+}-containing medium while no significant difference was seen in glutamate release by *Nlrx1*$^{-/-}$ astrocytes. Interestingly, the same observation of increased glutamate release by WT rat cortical astrocytes after Ca^{2+} removal was reported before by Kostic and colleagues. They explained this phenomenon by the fact that Ca^{2+} removal from the extracellular space stimulates Ca^{2+} release from the intracellular Ca^{2+} stores, hence enhancing glutamate release (Kostic et al. 2017). Nevertheless, these findings do not explain the phenotype of *Nlrx1*$^{-/-}$ astrocytes' excess glutamate release, as there was no difference between their glutamate release in both media. This is probably because *Nlrx1*$^{-/-}$ astrocytes have already reached their maximum capacity of glutamate release and Ca^{2+} removal does not result in any additional effect. These findings suggest that glutamate release by *Nlrx1*$^{-/-}$ astrocytes is not mediated by Ca^{2+} entry from the culture medium.

4.1.2. NLRX1 inhibits Ca^{2+} release from the ER in astrocytes

Accumulating evidence suggests that glutamate release by astrocytes is mediated mostly by Ca^{2+} release from the ER in response to the GPCR stimulation followed by IP3 activation in astrocytes (Hamilton and Attwell 2010; Harada, Kamiya, and Tsuboi 2016; Hua et al. 2004; Mahmoud et al. 2019). In addition, using rat cortical astrocytes, Reyes and Parpura reported that mitochondrial Ca^{2+} also plays a role in modulating $[Ca^{2+}]_i$ level, and thus modulates glutamate release by astrocytes. When they inhibited Ca^{2+} release from the mitochondria by CsA, they observed a significant reduction in glutamate release. Contrariwise, when they stimulated Ca^{2+} release from the mitochondria by FCCP, there was a remarkable increase in the astrocytic glutamate release (Reyes and Parpura 2008).

In our experiments, after excluding the role of the extracellular Ca^{2+}, we investigated whether Ca^{2+} release from the intracellular Ca^{2+} stores, mitochondria and ER, mediates the excess glutamate release from *Nlrx1*$^{-/-}$ astrocytes. We incubated WT and *Nlrx1*$^{-/-}$ astrocytes with 2-APB (inhibitor of Ca^{2+} release from the ER) or CsA (inhibitor of Ca^{2+} release from the mitochondria). Treating astrocytes with 2-APB resulted in a significant reduction in glutamate release from *Nlrx1*$^{-/-}$ astrocytes relative to WT, while no significant change was seen in glutamate release from CsA-treated astrocytes between both genotypes. These data suggest that excess glutamate release from *Nlrx1*$^{-/-}$ astrocytes is mediated by excess Ca^{2+} release from the ER. These observations support the previous reports that ER is the main source of increased $[Ca^{2+}]_i$ level in the mechanism of Ca^{2+}-mediated exocytosis in astrocytes (Hamilton and Attwell 2010; Harada et al. 2016; Hua et al. 2004; Mahmoud et al. 2019).

4.1.3. NLRX1 inhibits glutamate exocytosis by astrocytes

As we showed that excess glutamate release from *Nlrx1*$^{-/-}$ astrocytes is induced by excess Ca^{2+} release from the ER, finally, we evaluated whether it is mediated by exocytosis. We measured the mRNA expression of the proteins involved in exocytosis in WT and *Nlrx1*$^{-/-}$ astrocytes. *Nlrx1*$^{-/-}$ astrocytes showed significantly higher mRNA expression of the vesicular compartments of exocytosis downstream of Ca^{2+} release from the ER. These proteins include the astrocytic Ca^{2+} sensor, synaptotagmin 11, and the vesicular fusion proteins, VAMP2 and VAMP3. However, there was no significant difference in the mRNA expression of the cell membrane fusion proteins or proteins of exocytosis upstream of Ca^{2+} release from the ER. These observations suggest that exocytosis is significantly enhanced in *Nlrx1*$^{-/-}$ astrocytes relative to WT.

Together these data suggest that NLRX1 inhibits glutamate release from astrocytes by suppressing Ca^{2+} release from the ER, and consequently suppressing Ca^{2+}-mediated glutamate exocytosis.

Two previous studies from the same group, using rat cortical astrocytes and brain tissue slices in addition to human astrocyte cultures from embryonic stem cells, demonstrated that inflammatory mediators, such as PGE2 and TNF-α, enhance Ca^{2+}-mediated glutamate exocytosis by astrocytes in many CNS diseases (Bezzi et al. 1998, 2001). However, nothing is known about the regulatory factors of exocytosis under physiological conditions. To the best of our knowledge,

we report for the first time that NLRX1 regulates Ca^{2+}-mediated glutamate exocytosis in healthy astrocytes.

4.2. Effect of NLRX1 on glutamate uptake by astrocytes

Glutamate uptake by astrocytes is one of the extensively studied functions of astrocytes in the CNS. The importance of this astrocytic function is highlighted by its implication in preventing glutamate excitotoxicity. Also, by converting glutamate to glutamine, astrocytes provide neurons with a precursor for active neurotransmitters' synthesis. Till now, we know a lot about its mechanism, mediated by the glutamate transporters, EAAT1 and EAAT2 (Anderson and Swanson 2000; Mahmoud et al. 2019). In addition, extensive studies focused on the regulation of astrocytic glutamate uptake. Various factors were reported to affect glutamate transporters' expression, translation, post-translational modifications, and functional activity (Chi-Castañeda et al. 2017; Gegelashvili and Schousboe 1997; Mahmoud et al. 2019; Sattler and Rothstein 2006).

In our study, we uncovered the role of NLRX1 on astroglial glutamate uptake. We incubated WT and *Nlrx1*$^{-/-}$ astrocytes with different concentrations of glutamate, and we measured how much astrocytes uptake from this extracellular glutamate. With the low concentration of glutamate, we did not see a significant difference in the glutamate uptake between both genotypes. However, when we increased glutamate concentration, WT astrocytes tripled the amount of glutamate they uptake/µg of protein. This observation agreed with the previous report published by Abe et al. They incubated rat cortical astrocytes with different concentrations of glutamate for different incubation periods. They reported that astrocytes increased their glutamate uptake capacity in a concentration and time-dependent manner (Abe, Abe, and Saito 2000). By contrast, *Nlrx1*$^{-/-}$ astrocytes did not show any significant difference in their glutamate uptake between both concentrations of glutamate. This created a fivefold increase in the glutamate uptake by WT astrocytes compared to *Nlrx1*$^{-/-}$ with the high concentration of glutamate.

4.2.1. NLRX1 does not influence the protein expression of glutamate transporters

Knowing that glutamate uptake by astrocytes is mediated by the glutamate transporters, GLT-1 and GLAST (Eulenburg and Gomeza 2010; Lehre and Danbolt 1998), we measured their mRNA and protein expression in the astrocyte cultures of both genotypes. Surprisingly, the mRNA expression of both transporters was significantly higher in *Nlrx1*$^{-/-}$ astrocytes compared to WT,

which negatively correlates with our results of their glutamate uptake. By contrast, there was no significant difference in the total protein expression or the cell surface expression of both GLT-1 and GLAST between both groups of astrocytes.

Several possible explanations may interpret this lack of correspondence between the mRNA and protein expressions of glutamate transporters in *Nlrx1*$^{-/-}$ astrocytes (Greenbaum et al. 2003; Liu, Beyer, and Aebersold 2016). One possibility could be related to the various post-transcriptional modifications occurring between the gene transcription and protein synthesis. These modifications include activation or inhibition of many signaling pathways, such as PKA, PKC, PI3K, and NF-κB (Chi-Castañeda et al. 2017; Gegelashvili and Schousboe 1997; Sattler and Rothstein 2006). Depending on the interactions with different environmental factors and other signaling pathways, activation of NF-κB could mediate either upregulation or downregulation of the protein expression of GLT-1 and GLAST (Chi-Castañeda et al. 2017; Gegelashvili and Schousboe 1997; Sattler and Rothstein 2006). Taking in consideration that NLRX1 is known to inhibit NF-κB pathway (Allen et al. 2011; Ma et al. 2019; Theus et al. 2017), it is possible that loss of the brakes on NF-κB activation reduces GLT-1 and GLAST protein expression relative to their mRNA expression in *Nlrx1*$^{-/-}$ astrocytes. The second possible cause might be the post-translational modifications that reduce the protein half-life or induce its degradation, such as protein glycosylation, phosphorylation or oxidation (Greenbaum et al. 2003; Liu et al. 2016). Several reports demonstrated that protein oxidation by ROS results in protein damage and degradation (Davies, Lin, and Pacifici 1987; Pajares et al. 2015). This might be applicable in our case, as we showed later in our results that *Nlrx1*$^{-/-}$ astrocytes have more ROS activity than WT. In this regard, the effect of NF-κB activation and oxidative stress on the GLT-1 and GLAST proteins' translation and post-translational modifications in *Nlrx1*$^{-/-}$ astrocytes remain to be determined.

Nevertheless, these data suggest that NLRX1-mediated increased glutamate uptake cannot be attributed to transcriptional or translational modifications of glutamate transporters in astrocytes.

In agreement with our findings, various factors were reported to influence glutamate uptake by astrocytes with no effect on the mRNA or protein expression of the transporters. Using *Xenopus* oocytes and human embryonic kidney cells (HEK293) expressing the cloned GLAST-1 cDNA, Conrad and Stoffel reported that direct phosphorylation of GLAST protein by PKC reduced its glutamate uptake function. However, immunofluorescence staining did not show any difference

between the expression of the phosphorylated and the non-phosphorylated forms of the protein (Conradt and Stoffel 1997). H_2O_2 also inhibits the function of both transporters by direct oxidation of the sulfhydryl group (SH) of both transporter proteins in rat cortical astrocytes (Trotti et al. 1997), without any effect on their mRNA or protein expression evidenced by qPCR, immunoblotting, and immunocytochemical techniques (Miralles et al. 2001). In another study, arachidonic acid was shown to reduce the functional activity of EAAT1 by decreasing its affinity to glutamate and its maximum glutamate transport rate, with no effect on its protein expression. The results in this study were obtained from HEK293 Cells and *Xenopus* oocytes transfected with EAAT1 – 3 (Zerangue et al. 1995).

4.2.2. NLRX1 enhances mitochondrial functions and the functional activity of glutamate transporters in astrocytes

The presence of a sufficient amount of intracellular ATP in astrocytes is mandatory to maintain normal functions of the glutamate transporters (Pellerin and Magistretti 1997; Sibson et al. 1998). Previous studies reported that brain ischemia is associated with reduced or depleted intracellular ATP, thus accompanied by glutamate uptake failure and even reversal of the transporters in severe cases (Grewer et al. 2008; Rossi et al. 2000). In addition, a significant part of the sequestrated glutamate in astrocytes is metabolized to α-ketoglutarate, which acts as a substrate for ATP production (McKenna 2013). Therefore, we were interested to know if NLRX1 affects the level of intracellular ATP in astrocytes. We measured the level of intracellular ATP in WT and *Nlrx1*$^{-/-}$ astrocytes. As we expected, *Nlrx1*$^{-/-}$ astrocytes had 20% less intracellular ATP than WT, which could either be a cause or a result of the reduced glutamate uptake in these cells. Nonetheless, these data suggest that NLRX1 enhances intracellular ATP production in astrocytes.

Furthermore, oxidative stress, specifically by H_2O_2, reduces the functional activity of the transporters in astrocytes (Sorg et al. 1997; Volterra et al. 1994) by direct oxidation of the SH group of both transporter proteins (Trotti et al. 1997). Therefore, we measured ROS activity in both WT and *Nlrx1*$^{-/-}$ astrocytes. Interestingly, we found that *Nlrx1*$^{-/-}$ astrocytes have about 50% more oxidative activity than WT, which may explain the significant reduction of their glutamate uptake activity.

Similar to our obtained results from NLRX1, previous studies showed that many other factors regulate the function of the transporters indirectly by modulating the level of oxidative activity in

astrocytes. In a study on brain tissues from patients with AD, amyloid-β peptide induced oxidative stress in astrocytes and resulted in a reduction of glutamate uptake by astrocytes (González-Reyes et al. 2017). In another study, treating human astrocyte cultures from fetal brain tissue with IL-1β and IFN-ɣ pro-inflammatory cytokines induced ROS production by astrocytes and reduced their function of glutamate uptake (Sheng et al. 2013).

Collectively, these results corroborate the data obtained from 2 independent research groups that NLRX1 enhances ATP production and inhibits oxidative stress in 2 different cell types, renal tubular epithelial cells (Stokman et al. 2017) and human breast cancer cells (Singh et al. 2019).

Moreover, we measured mtDNA as an indication of the number of mitochondria, as previously described (Fuke et al. 2011). We did not find any significant difference in the amount of mtDNA between WT and *Nlrx1*$^{-/-}$ astrocytes. Therefore, we concluded that NLRX1 does not increase the number of mitochondria in astrocytes.

When we incubated our cultures with 100 μM of glutamate, we did not see a significant difference in glutamate uptake between WT and *Nlrx1*$^{-/-}$ astrocytes, probably because the functional capacity of glutamate transporters in *Nlrx1*$^{-/-}$ astrocytes was enough to uptake such low concentration of glutamate.

With 200 μM of glutamate, glutamate transporters need to increase their energy consumption and functional capacity to uptake such high concentration of glutamate, which was the case for WT astrocytes. However, probably because of the reduced intracellular ATP and increased ROS activity in *Nlrx1*$^{-/-}$ astrocytes, glutamate transporters could not increase their functional activity to uptake more glutamate and that is why we saw this significant difference between WT and *Nlrx1*$^{-/-}$ astrocytes' glutamate uptake only with the high concentration of glutamate.

Taken all together, our findings suggest that NLRX1 enhances astroglial glutamate uptake by maintaining normal functional activity of the astrocytic glutamate transporters, GLT-1 and GLAST. This is achieved by damping intracellular oxidative stress and, at least partially by enhancing mitochondrial ATP production in astrocytes.

4.3. How NLRX1 modulates the functions of the mitochondria and the ER

The connection between these two mechanisms by which NLRX1 regulates astrocytic glutamate uptake and release is still unknown. Through its localization in the mitochondria, it is plausible that NLRX1 enhances mitochondrial functions. However, we report for the first time that

NLRX1 mediates an effect on the ER. Taking into consideration that the exact location of NLRX1 in the mitochondria is still under debate, in which one research group reported that it is located in the outer mitochondrial membrane, based on immunofluorescence staining of HELA cells transfected with NLRX1-GFP (Moore et al. 2008). Using similar imaging techniques, three other groups showed that it is situated in the mitochondrial matrix (Arnoult et al. 2009; Rhee et al. 2013; Singh et al. 2018). *In vivo* studies on *Nlrx1-KD* mice showed that NLRX1 also interacts with TRAF6 and IKK in the cytoplasm to inhibit NF-κB signaling pathway (Allen et al. 2011; Xia et al. 2011). It might be possible that NLRX1 is situated at the ER/mitochondrial transitions.

The ER/mitochondrial transitions or contacts, also known as the mitochondria-associated membrane (MAM), are sites at which the outer mitochondrial membrane and the ER surface run in parallel at a constant distance ranging from 10 to 80 nm, as shown by the electron microscopy (Ilacqua et al. 2017). These contact sites were first isolated in 1990 (Vance 1990) and were visualized by fluorescence, later in 1998 (Rizzuto et al. 1998). Now, visualization of these contact sites is much improved thanks to the great microscopic technologies such as electron microscopy, electron tomography, and wide-field digital 3D deconvolution microscopy. Proteins expressed at both membranes of the MAM interact together to regulate many functions between both organelles. In case of ER stress, the chaperone glucose-regulated protein 75 (GRP75) and transglutaminase type 2 (TG2) induce Ca^{2+} transfer from the ER to the mitochondria, which stimulates the opening of mitochondrial transition pores to initiate apoptosis (D'eletto et al. 2018; Deniaud et al. 2008; Honrath et al. 2017; Malhotra and Kaufman 2011). Other proteins expressed at these sites include Mitofusin 2 (MFN2) which induces mitochondrial fusion, proteins involved in innate immune signalings such as MAVS, STING, and the inflammasome receptor NLRP3, AKT protein involved in autophagy/mitophagy, in addition to many various proteins involved in Ca^{2+}, lipid, and ER homeostasis (Ilacqua et al. 2017). Dysregulation of these sites of ER/mitochondrial contacts in the CNS was shown to be associated with many diseases such as ALS. Oxidative stress and disturbance in Ca^{2+} homeostasis and protein folding in ALS are characteristic features of its neurodegeneration (Tadic et al. 2014).

We showed in our study that NLRX1 modifies the functions of both, the ER and mitochondria. NLRX1 also interacts with other proteins at the ER/mitochondrial transitions such as MAVS (Allen et al. 2011; Moore et al. 2008) and STING (Dempsey 2016; Deng et al. 2017; Haitao Guo et al. 2016). For these reasons, we think that NLRX1 might be situated in the MAM and executes

its functions on both organelles. However, the exact localization of NLRX1 and the mechanism by which it inhibits Ca^{2+} release from the ER require more in-depth investigations.

4.4. Conclusion and future perspectives

In our work, we provide evidence that NLRX1 enhances astroglial glutamate uptake by enhancing mitochondrial functions in astrocytes, that in turn promote the functional activity of glutamate transporters. NLRX1 also inhibits astroglial glutamate release by suppressing its Ca^{2+}-mediated exocytosis. By regulating these two astroglial functions, NLRX1 maintains glutamate homeostasis in the CNS and represents a potential therapeutic target for CNS diseases associated with glutamate excitotoxicity.

To our knowledge, this is the first time to be reported that one protein can regulate both processes, and therefore, it regulates glutamate homeostasis in the CNS.

As a continuation of the current work, further investigations on the exact location of NLRX1 in the mitochondria and the mechanism by which NLRX1 modulates Ca^{2+} release from the ER is essential to understand better how NLRX1 regulates glutamate release by astrocytes.

As a first step, we obtained our results from *in vitro* experiments on healthy mouse astrocyte cultures. As a second step, it would be necessary to confirm the role of NLRX1 in glutamate homeostasis in healthy CNS in different animal models. Future studies should also evaluate the role of NLRX1 in glutamate excitotoxicity in various animal models of inflammatory and neurodegenerative diseases in the CNS such as brain trauma, CNS infections, ALS, and MS.

As a recently discovered molecule, most of the obtained data on NLRX1 were based on *in vitro* studies using cell lines or primary animal cell cultures, and *in vivo* experiments on *Nlrx1*$^{-/-}$ versus WT mice. However, very minimal or limited studies involved the role of NLRX1 in human diseases. These studies included the expression of NLRX1 in different human diseases such as COPD (Kang et al. 2015), ATN and ACR (Stokman et al. 2017), and the *nlrx1* mutation associated with CHB virus infection susceptibility (Zhao et al. 2012). There is only one study in the CNS evaluated the *nlrx1* gene expression in patients with ruptured brain aneurysms (Theus et al. 2017). Based on these data, future studies should be directed more towards the role of NLRX1 in human diseases inside and outside the CNS. As a continuation of our study, the effect of NLRX1 on glutamate uptake and release should be evaluated in human astrocytes. The level of expression of NLRX1 should be determined in patients with various CNS diseases. Attention should be payed

more towards the study of the protective role that NLRX1 may play against the development and progression of these diseases specially those associated with glutamate excitotoxicity such as neurodegenerative diseases and MS.

All the currently used treatments for glutamate excitotoxicity reduce the toxic effects of glutamate on the postsynaptic neurons. Among these treatments, riluzole, which blocks glutamate neurotransmission in the CNS and blocks NMDA receptors in the postsynaptic neurons (Doble 1996). Memantine is another anti-glutamatergic drug that acts as a non-competitive NMDA receptor antagonist. It is currently used for treatment of moderate to severe Alzheimer's disease (Alam et al. 2017). NLRX1, as a regulator of glutamate homeostasis, enhances glutamate uptake and inhibits glutamate release by astrocytes. Therefore, it represents a potential novel therapy that not only prevents the toxic effect of glutamate on neurons, but it inhibits the onset or the primary causes of excitotoxicity. As a long-term perspective, future studies should focus on the possibility to use NLRX1 as a therapeutic target for CNS diseases associated with glutamate excitotoxicity.

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